Rescue of Hypercholesterolemia-Related Impairment of Angiogenesis by Oral Folate Supplementation

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OBJECTIVES
We examined whether oral folate supplementation would rescue a hypercholesterolemia (HC)-related impairment of ischemia-induced angiogenesis.

BACKGROUND
Folate protects against endothelial dysfunction, but the effect of folate supplementation on angiogenesis is little known.

METHODS
Sprague-Dawley rats were divided into four groups. Control rats were fed a normal diet (n = 18); HC rats (n = 18) were fed 2% cholesterol diet; and HC + folate (HC + F) rats were fed an HC diet with oral folate (0.003% in water). The left femoral artery and vein were surgically excised, and angiogenesis in the ischemic limb was evaluated. We also examined the effects of Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase, on angiogenesis in the HC + F state.

RESULTS
Laser Doppler blood flow (LDBF) analysis showed lower ischemic/normal LDBF ratio in the HC group than in the control group. Angiographic and histologic analyses on day 14 revealed a smaller angiographic score (p < 0.001) and capillary density (p < 0.001) in the HC group than in controls, which were associated with reduced tissue NOx and cyclic guanosine monophosphate (cGMP) levels. The LDBF ratio, angiographic score, and capillary density were significantly restored in the HC + F group (p < 0.01 vs. HC), which were associated with increased serum folate and tissue NOx and cGMP levels. Finally, L-NAME treatment abolished the beneficial action of folate on angiogenesis in the HC state.

CONCLUSIONS
Ischemia-induced angiogenesis was inhibited by HC, which was rescued by oral folate supplementation, at least in part, via an NO-dependent manner. (J Am Coll Cardiol 2003; 42:364–72) © 2003 by the American College of Cardiology Foundation

Enhancement of collateral vessel formation and angiogenesis in patients with peripheral arterial disease is an important strategy for minimizing ischemia-induced tissue injury, termed “therapeutic angiogenesis” (1). Neovascularization is stimulated by various cytokines to grow into ischemic foci, in which migration and proliferation of endothelial cells are essential events. We and other investigators (2,3) reported that endothelium-derived nitric oxide (EDNO) is an important regulator for angiogenesis. For example, angiogenesis induced by vascular endothelial growth factor was attenuated by inhibitors of nitric oxide synthase (NOS) (2). We also showed that ischemia-induced angiogenesis was reduced in mice lacking the gene for endothelial nitric oxide synthase (eNOS) (3).

Hypercholesterolemia (HC) is an established risk factor for atherothrombotic disease and is associated with endothelial dysfunction as characterized by impaired endothelium-dependent vasorelaxation (i.e., EDNO release) (4). The adverse effects of HC on vascular function were recently extended into the process of angiogenesis (5), which is mediated by a reduced endogenous EDNO formation (6).

Studies showed that folate and its active metabolite 5-methyltetrahydrofolate caused a reversion of HC-induced endothelial dysfunction through enhancement of nitric oxide (NO) production by regenerating tetrahydrobiopterin (BH4), a cofactor for NOS (7–11). Despite these beneficial actions of folate on the endothelium, the effects of oral folate supplementation on angiogenesis are little known. Accordingly, the present study was designed to test whether oral supplementation of folate would rescue the HC-related impairment of angiogenesis in a rat model of hindlimb ischemia.

METHODS
Experimental protocol. We employed a rat model of unilateral hindlimb ischemia as described previously (6,12). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine. Male Sprague-Dawley rats (n = 54) were randomly divided into three groups. Control (n = 18) rats were fed a normal diet and tap water; HC rats (n = 18) were fed a 2% cholesterol diet and tap water; hypercholesterolemia plus folate (HC + F) rats (n = 18) were fed the HC diet and drinking water containing folate (0.003 vol%). Two weeks later, all rats were subjected to unilateral hindlimb ischemia by removing the left femoral artery and...
vein (6,12). From the day of surgery until the end of the protocol, the HC+F rats continued to receive folate. The oral dose of folate was chosen according to a previous study (13), and this regimen (5 mg/kg/day) effectively increased serum levels of folate. Before and at days 7, 14, and 28, systemic blood pressure and heart rate were measured in the conscious state using a tail-cuff method (TK-370C, UNI-COM, Tokyo, Japan).

**Laser Doppler blood flow analysis.** We measured the ratio of ischemic (left)/normal (right) hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (moorLDI, Moor Instrument, Wilmington, Delaware) as described previously (6,12,14). At six time points (before and at days 1, 7, 14, 21, and 28), we performed laser Doppler scanning over the regions of interest (legs and feet). The average perfusion of the ischemic (left) and nonischemic (right) hindlimbs was computed, and blood flow was expressed as the ischemic (left)/normal (right) hindlimb blood flow ratio.

**Angiographic score.** At day 14, under pentobarbital anesthesia, a 26-gauge catheter was inserted into the abdominal aorta of control (n = 6), HC (n = 6), and HC+F (n = 6) rats. Hindlimbs were perfused with 10 mL of warm saline containing heparin (10 U/ml) at 90 mm Hg of perfusion pressure. After animals were euthanized by a high dose of pentobarbital, postmortem hindlimb angiography was performed as described previously (6). The medial thigh adductor muscle area of the ischemic limb was selected to quantify the developed collateral vessels (i.e., angiographic score). Angiographic scores were calculated as described previously (6).

**Capillary density.** An additional six animals in each group were euthanized at day 14, and ischemic (left) and nonischemic (right) hindlimb skeletal muscles (quadriceps muscles) were harvested and fixed in methanol. Tissues were embedded in paraffin, and 5-μm-thick sections were prepared. We used a monoclonal antibody directed against von Willebrand Factor (F8/86, DAKO, Glostrup, Denmark) as a marker of endothelium. Capillary endothelial cells positively stained with this monoclonal antibody were counted using light microscopy, and the capillary densities of both ischemic and nonischemic hindlimb skeletal muscles were calculated and expressed as the number of capillaries/high power field (×400) (12). To ensure that analysis of capillary density was not overestimated as a result of muscle atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was also determined.

**Tissue contents of cyclic guanosine monophosphate (cGMP) and nitrate + nitrite (NOx).** The medial thigh adductor muscles were harvested from the ischemic hindlimb of six rats in each group at day 14. The tissue samples were weighed, snap-frozen in liquid N2 and stored at −80°C. The assay for cyclic guanylate monophosphate (cGMP) was performed as described previously (3). Tissue NOx content was measured by a high-performance liquid chromatography as described (6).

**Additional biochemical analysis.** Plasma and tissue levels of NOx were measured by high-performance liquid chromatography (15). Plasma levels of homocysteine, folate, and vitamin B12 were also measured by high-performance liquid chromatography as described elsewhere (16–18). Serum concentrations of total- and high-density lipoprotein cholesterol were determined enzymatically using commercially available kits (Boehringer Diagnostica, Mannheim, Germany, and Wako Chemicals, Richmond, Virginia).

**Effects of NO inhibition on the folate-mediated modification of angiogenesis.** To examine whether the effects of folate on angiogenesis is related to in vivo NO bioactivity, we tested the effects of oral administration of NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), on folate-mediated modification of angiogenesis. In an additional group of rats (n = 12), HC+F diet was started at 14 days before limb ischemia surgery. On the day of surgery, oral L-NAME administration (1.0 mg/ml in drinking water) was started, and LDBF and angiographic score were analyzed thereafter. The dose of L-NAME was determined according to a previous study (19), and this regimen effectively inhibited endogenous NO bioactivity (19).

**Reagents.** All reagents were purchased from Sigma unless otherwise specified. The immunostaining kit (VECTASTAIN) including the secondary anti-mouse IgG polyclonal antibody was purchased from Vector Laboratories.

**Statistical analysis.** Results are all expressed as means ± SE. Comparisons were performed by one-way analysis of variance, which were followed by the Fisher least significant difference test for comparisons between any two groups. Statistical significance was assumed at p < 0.05.

**RESULTS**

Table 1 summarizes the mean body weight, heart rate, and systolic arterial blood pressure of the four experimental groups measured before surgery and at postoperative days 7, 14, and 28. Body weight was higher in the HC and HC+F groups than in the control group at days 7, 14, and 28. There were no significant differences in body weight between the HC and HC+F groups at any time point, indicating that supplemental oral folate did not affect body weight. There were no significant differences in heart rate...
nor in systolic blood pressure among the groups at any time points examined (Table 1).

**Serum cholesterol and plasma folate levels.** Table 2 summarizes serum cholesterol and plasma folate levels at postoperative day 14. The plasma folate level significantly increased in the HC+F group compared with the control and HC groups. Serum total-cholesterol modestly but significantly increased in the HC and HC+F groups compared with the control group. Serum high-density lipoprotein cholesterol significantly decreased in the HC and HC+F groups compared with the control group. There were no significant differences in total or high-density lipoprotein cholesterol levels between the HC and HC+F groups, indicating that oral folate supplementation did not affect serum cholesterol levels under the HC condition.

**Laser Doppler analysis for hindlimb blood flow.** Figure 1A shows representative images of the hindlimb blood flow as assessed by laser Doppler at postoperative days 7, 14, and 28. Serial LDBF analyses revealed a progressive recovery of the blood flow within 28 days after induction of hindlimb ischemia in the control group. The recovery of blood flow in the ischemic hindlimb was significantly impaired in the HC group. However, oral folate supplementation (HC+F group) contributed to the recovery of blood flow in the ischemic hindlimb. Before surgery, the ratio of the ischemic (left)/normal (right) hindlimb LDBF was approximately 1.0 in all three groups (Fig. 1B), indicating no differences in blood flow between right and left legs. Immediately after surgical induction of left limb ischemia, the ischemic/normal LDBF ratio markedly decreased to almost 0.4 in all three groups. These values did not differ among the groups, indicating that the severity of the induced limb ischemia was comparable among the groups.

During the follow-up period, the ischemic/normal LDBF ratio was significantly lower in the HC group than in the control group at postoperative days 7, 14, 21, and 28. However, the recovery of the ratios was much greater in the HC+F group than in the HC group at these time points (Fig. 1B).

**Iliac arteriography and determination of the angiographic score.** To examine whether the reduced LDBF ratio in the HC group was associated with a decrease in the angiographically visible collateral vessel formation (angiogenesis), we performed postmortem angiography at postoperative day 14. There were many collateral vessels issuing from the internal iliac artery in the ischemic medial thigh in the control group. In contrast, the HC group had fewer collateral vessels in this area. The HC+F group, however, revealed an enhanced formation of collateral vessels compared with the HC group (Fig. 2A). The mean angiographic score on postoperative day 14 was significantly lower in the HC group than in the control group, whereas it was restored in the HC+F group almost to the level of the control group (Fig. 2B).

**Tissue capillary density.** Using an anti-von Willebrand factor monoclonal antibody, we performed immunohisto-

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### Table 1. Baseline Data of Animals

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<th>Group</th>
<th>BW (g)</th>
<th>HR (beats/min)</th>
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**Footnotes:**
- *p < 0.05 vs. control.
- †p < 0.01 vs. control.
chemical staining for the capillary endothelial cells in both ischemic and nonischemic hindlimb tissues at postoperative day 14. Quantitative analysis in the ischemic hindlimb tissues revealed a significant decrease in the capillary density in the HC group compared with the control group. However, the capillary density was significantly increased in the HC+F compared with the HC group. Similarly, the capillary/muscle fiber ratio, which can exclude the effects of interstitial edema or muscular atrophy on capillary counts, was decreased in the HC group and was restored in the HC+F group. In the nonischemic hindlimb tissues, the capillary density, and capillary/muscle fiber ratio did not differ among the three groups (Fig. 3).

**Plasma levels of NOx and tissue contents of NOx and cGMP.** The plasma NOx level significantly decreased in the HC group compared with the control group (Table 2). Serum vitamin B_{12} level did not differ among the three groups (data not shown). We further analyzed the tissue contents of cGMP and NOx in the ischemic hindlimb (n = 6 in each group). Tissue NOx and cGMP contents significantly decreased in the HC group as compared with the control group, while they were restored in the HC+F group (Table 2).

**Plasma level of homocysteine.** The plasma level of homocysteine significantly increased in the HC group compared with the control group (p < 0.001), while it was restored in the HC+F group to a level similar to that of the control group (p < 0.001 vs. HC) (n = 6 in each group) (Table 2).

**Effects of NO inhibition on folate-modification of angiogenesis in the HC state.** Because ischemia-induced angiogenesis depends on EDNO formation, we examined the effects of L-NAME on angiogenesis in the HC+F group. Figure 4A shows the time course of the ischemic/normal hindlimb LDBF ratio. Folate treatment restored the LDBF ratio in the HC state. However, co-administration of L-NAME significantly suppressed the LDBF ratio to a level much lower than that in the HC group. Figure 4B shows the angiographic score examined on postoperative day 14. Again, folate treatment restored the angiographic score of the HC state. However, co-administration of L-NAME markedly suppressed the score, which was lower than that in the HC group.

**DISCUSSION**

The present study demonstrated that ischemia-induced angiogenesis was impaired by HC, a major risk factor for atherosclerosis, in a rat model of hindlimb ischemia. The impaired angiogenesis in HC rats was associated with reduced plasma NOx, tissue NOx, and cGMP levels, indicating reduced endogenous NO bioactivity. Importantly, the present study for the first time demonstrated that the HC-related impairment of angiogenesis was restored by oral supplementation of folate. The rescue effect was, at least in part, related to increased NO bioactivity because L-NAME completely suppressed the effects of folate on angiogenesis.

**Mechanisms for the HC-related impairment of angiogenesis.** There are several possible mechanisms for the HC-related impairment of angiogenesis. First, HC-mediated endothelial dysfunction may account for the major part of the impaired angiogenesis. It has been shown that HC impairs endothelial function, including EDNO formation (4), and EDNO is a critical regulator for angiogenesis (2,3). For example, we showed that ischemia-induced angiogenesis was markedly attenuated in mice lacking the eNOS gene (3). Endothelium-derived NO also supports endothelial migration (20). Therefore, impaired EDNO formation in dysfunctional endothelium during HC may, at least in part, account for the impaired angiogenesis. Indeed, both plasma and tissue levels of NOx and cGMP, a second messenger of NO, were significantly decreased in the HC group compared with controls in the present study, indicating a reduced NO bioavailability in HC.

Second, eNOS has been shown to produce not only NO but also superoxide radical (21). Under physiologic conditions, eNOS produces NO. However, it produces superoxide anion under dyslipidemic conditions, resulting in an increase of endothelial oxidative stress in the HC state (22,23). Moreover, HC itself may have directly inhibited the migration and/or proliferation of endothelial cells. A recent study demonstrated that oxidized low-density lipoprotein inhibited endothelial migration (24). Chen et al. (25,26) also showed that ex vivo angiogenesis from explants of human and rabbit coronary artery segments was significantly impaired in HC compared with healthy subjects. Likewise, superoxide anion directly deteriorates endothelial...
Figure 1. (A) Representative laser Doppler blood flow (LDBF) images at days 7, 14, and 28 are shown. (B) Before and after surgery, the hindlimb LDBF ratio did not differ among the three groups. The control group revealed progressive recovery of the ischemic/normal LDBF ratio within 28 days after induction of limb ischemia. However, the LDBF ratio was lower in the hypercholesterolemia (HC) group than in the controls. In the HC + Folate group, the LDBF ratios were significantly improved compared with the HC group; n = 6 in each group. *p < 0.05; **p < 0.01 vs. control; †p < 0.05; ††p < 0.01 vs. HC.
Figure 2. (A) Representative postmortem angiograms taken at day 14 are shown. There are numerous collateral vessels in the medial thigh area in the control group. However, the hypercholesterolemia (HC) group showed reduced collateral vessels. Folate supplementation (HC + Folate group) increased collateral vessels. (B) The angiographic score was significantly lower in the HC than in the control group, which was rescued in the HC + Folate group. n = 6 in each group; ***p < 0.001.
proliferation (27). Taken together, endothelial dysfunction with decreased EDNO formation and increased superoxide releases are probably responsible for the impaired angiogenesis in the HC state.

Folate restored ischemia-induced angiogenesis in HC. Recent studies indicate that a low level of plasma folate is associated with the increased risk of cardiovascular disease (28). Conversely, folate supplementation preserved endothelial function in patients with cardiovascular risk factors (7–11). The present study is the first to show that oral folate administration has favorable impacts on ischemia-induced angiogenesis. Folate supplementation augmented both angiographically visible (diameter >200 μm) collateral vessel formation (i.e., arteriogenesis) and histologic capillary vessel formation (i.e., angiogenesis) (29). There may be several possible mechanisms for the beneficial actions of folate on arteriogenesis/angiogenesis. First, folate may have restored endothelial function via a regeneration of BH4 because folate and its active metabolite metabolite 5-methyltetrahydrofolate are involved in the synthesis of BH4 that is an essential cofactor for eNOS (7–9). In fact, the ability of eNOS to produce superoxide anion depends on the deficiency of BH4 (21). Thus, regeneration of BH4 by folate supplementation may have resulted in the reduced amount of superoxide anion and enhanced NO formation. This hypothesis is further supported by the present findings that HC suppressed tissue NOx and cGMP contents, whereas folate supplementation restored the levels of these molecules.

In the present study, treatment with L-NAME completely blocked the beneficial effects of folate on angiogenesis in the HC state. Both ischemic/normal LDBF ratio and angiographic score (arteriogenesis) were suppressed by L-NAME down to levels even lower than those observed in the HC group. These indicate that not only angiogenesis but also arteriogenesis depends on basal EDNO bioactivity. Consistently, Matsunaga et al. (30) reported that arteriogenesis in the ischemic tissues depends on vascular endothelial growth factor-mediated endothelial NO release. We also previously reported that arteriogenesis was suppressed by hypercholesterolemia, which was rescued by L-arginine, a precursor of NO (6). Lloyd et al. (31) reported using a rat model of limb ischemia that, again, arteriogenesis depends
on NO. These studies collectively support that EDNO is an important factor for angiographically visible arteriogenesis.

Second, folate may have restored endothelial function via a homocysteine-dependent pathway. Studies showed that HC was associated with hyperhomocysteinemia (32), an atherothrombotic risk factor that inhibits endothelial function (33,34). Folate is involved in the metabolic pathway of homocysteine, and folate supplementation has been shown to reduce plasma homocysteine levels (13). A recent study indeed showed that folate supplementation ameliorated hyperhomocysteinemia-related renal ischemia (35). The present study showed that plasma homocysteine concentrations were increased in the HC group compared with the control group, while oral folate supplementation reduced plasma homocysteine concentrations to levels similar to those of the control group (Table 2). Thus, it is likely that folate reversed homocysteine-induced endothelial dysfunction, which may be an additional mechanism for the folate-mediated restoration of ischemia-induced angiogenesis in the HC state (36). Finally, folate supplementation did not affect serum total cholesterol or HDL-cholesterol levels. Therefore, the restoration of angiogenesis by oral folate is not likely to be mediated by alteration of serum lipid profiles.

**Study limitations.** In the present study, the LDBF ratio in the HC rats kept up with that of control rats in chronic phase. In this sense, our rat model of limb ischemia may not mimic patients with chronic peripheral-artery occlusive disease. One may consider that HC might have simply slowed the recovery of the LDBF ratio and folate accelerated the recovery process. Nevertheless, supplemental folate significantly improved angiogenesis at days 7 through 28 after ischemia, which is a critical time period for endothelial regeneration. Second, we administered 5 mg/kg/day of folate to each animal. This dose is almost identical to 250 mg/day in humans and is apparently high. Therefore, it is still unknown whether clinical doses of folate have similar efficacies in humans. Third, serum cholesterol levels in HC rats were relatively low compared with other studies. The reason is unknown, but our method of cholesterol measurements revealed a significant and definite difference between the control and HC groups. Therefore, high cholesterol diet at least induced an HC state in our rat model. Finally, although folate increased the number of capillaries in the ischemic tissues, the role of direct vasodilating action of folate on angiogenesis or arteriogenesis should be taken into account because folate can directly augment endothelium-dependent vasodilation.

**Conclusions.** Supplemental oral folate restored the impaired ischemia-induced angiogenesis in the HC state. The mechanism is likely to be related to the folate-mediated restoration of endothelial function and endogenous NO formation. Oral folate administration may be a feasible therapeutic means to increase angiogenesis and collateral vessel formation in patients with peripheral-artery occlusive disease complicated with dyslipidemia.

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