L-Arginine inhibits smooth muscle cell proliferation of vein graft intimal thickness in hypercholesterolemic rabbits

Jin Okazaki, Kimihiro Komori *, Katsumi Kawasaki, Daihiko Eguchi, Masaru Ishida, Keizo Sugimachi

Department of Surgery II, Faculty of Medicine, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812, Japan

Received 4 December 1996; accepted 10 July 1997

Abstract

Objective: The effect of the chronic administration of L-arginine on intimal thickness and the kinetics of smooth muscle cell proliferation in autovein grafts in hypercholesterolemic rabbits were examined. Methods: Male rabbits were fed a 1% cholesterol diet (control group) and a 1% cholesterol diet supplemented by 2.25% L-arginine HCl in drinking water (arginine group). Each group underwent reversed autologous vein bypass grafting of the left common carotid artery using the left external jugular vein. At 2 or 4 weeks after operation, intimal cell proliferation was determined by 5-bromo-2-deoxyuridine BrdU incorporation and intimal thickness of the graft was measured with an ocular cytometer. At 4 weeks after operation, endothelium-dependent responses were examined by isometric tension recording. Results: At 4 weeks after operation, the level of plasma arginine and citrulline are significantly higher in the arginine group compared with the control. Intimal thickness in the arginine group was significantly reduced, compared with that of the control at 4 weeks after operation. The BrdU labeling index of the control was significantly higher than that of the arginine group at 4 weeks after operation. ACh caused endothelium-dependent relaxation in the arginine group, while in the control group, ACh did not relax. Conclusions: These results suggest that smooth muscle cell proliferation of the rabbit jugular vein grafts during hypercholesterolemia occurs at an early stage after graft implantation, prior to the development of intimal thickness. Intimal thickness of vein graft during hypercholesterolemia was reduced by chronic administration of dietary L-arginine, by inhibiting smooth muscle cell proliferation. The enhancement of NO production in the blood vessel wall may therefore be useful for preventing late graft failure. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide; L-arginine; Vein graft; Hypercholesterolemia

1. Introduction

An autologous vein graft is the most suitable conduit for reconstruction at many sites of arterial occlusive disease [1]. In the early postoperative period after graft replacement, besides technical difficulties, acute platelet thrombosis may frequently lead to graft occlusion [2]. Late graft occlusion tends to be caused by either intimal thickness or a progression of the underlying atherosclerotic vascular disease [3,4].

Endothelium-derived relaxing factor (EDRF), which is now known as nitric oxide (NO) [5], has recently been shown to be not only a potent vasodilator but also to inhibit leukocyte–endothelial interaction [6,7], platelet adherence and aggregation [8–10] and vascular smooth muscle proliferation [11].

L-arginine is the precursor for the formation of NO [12,13]. The administration of L-arginine enhances NO production, which in turn reduces the development of proliferative atherosclerotic lesions [14] and intimal hyperplasia after balloon angioplasty [15]. Recently, Davies et al. [16] demonstrated that L-arginine reduced the degree of intimal thickness in experimental vein grafts.

Hypercholesterolemia is one of the most important risk factors for atherosclerosis [17,18] and it also accelerates graft failure [19,20]. We recently demonstrated that hypercholesterolemia accelerated intimal thickness in the rabbit.
femoral vein grafts [20]; this was due to the enhancement of cell proliferation. However, little information is available regarding the effect of L-arginine on either intimal thickness or smooth muscle cell proliferation induced by hypercholesterolemia. The present experiments were therefore designed to determine whether or not the chronic administration of L-arginine inhibits the intimal thickness of autologous vein grafts and reduces the cholesterol levels in hypercholesterolemic rabbits. In addition, the intimal cell proliferation determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation and the endothelium-dependent responses by isometric tension recording were also examined.

2. Methods

Male New Zealand White Rabbits (Body weight: 2.5–3.0 kg), which received 1% cholesterol chow (chow contains 1.12% L-arginine) were divided into two groups as follows: The control group received normal drinking water. The arginine group received 2.25% L-arginine (L-arginine, Sigma Chemical, St. Louis, MO) [14] supplementation in their drinking water (2.25 g/100 ml; for an average daily dose of 2 g/kg body weight). The above chow was fed 14 days before operation, and the arginine was administered 7 days before operation and thereafter both were continued until harvest.

Each group underwent a reversed autologous vein bypass grafting of the left common carotid artery with the left external jugular vein [21,22]. All animals were then killed with an overdose of pentobarbital sodium and the arterial vein bypass grafts were harvested 14 days or 28 days postoperatively.

All animal care complied with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (National Institute of Health, publication No. 85-23, revised 1985).

2.1. Operative procedures

Anesthesia was induced with ketamine hydrochloride (70 mg/kg, intramuscularly, Sigma Chemical, St. Louis, MO) and maintained with pentobarbital sodium (30 mg/kg, intravenously). The operation was performed with an operating microscope (Topcon, Tokyo, Japan) under sterile conditions. After a longitudinal neck incision, the left external jugular vein and the left common carotid artery were exposed. The branches of the jugular vein were ligated and then the vein was excised. After excision, the vein was kept moistened in heparinized saline solution. Heparin (200 units/kg) was administered intravenously. The carotid artery was clamped at the proximal and distal ends. A proximal longitudinal arteriotomy was made, and the jugular vein was reversed and anastomosed to the artery in an end-to-side manner by use of a continuous 10-0 monofilament nylon suture. Distal anastomosis was performed in a similar manner. The left common carotid artery was ligated and divided between the two anastomosis with 9-0 silk sutures, and then the wound was closed in layers.

2.2. Blood sample analysis

Blood was obtained from a cannula placed in the middle ear artery at the time of operation and at 2 or 4 weeks after operation before animals were killed under anesthesia. The concentration of the plasma arginine and plasma citrulline, which is a by-product of nitric oxide, was measured by reverse phase high-performance liquid chromatography [23]. The total plasma cholesterol levels were assayed spectrophotometrically using a commercially available kit (Type L Wako Cholesterol; Wako Chemicals, Japan).

In addition, the plasma cholesterol levels were assayed in the normolipidemic rabbits (n = 7).

2.3. Blood pressure measurement

At operation and 4 weeks after operation on harvest, the systolic blood pressure was measured through a cannula placed in the middle ear artery using a pressure transducer connected to a Polygraph 360 monitoring system (NEC Sanei, Tokyo, Japan). All measurements were made under anesthesia conditions.

2.4. Harvest of implanted grafts

At 2 weeks after operation, the rabbits in the control (n = 5) and L-arginine (n = 5) group were sacrificed. At 4 weeks after operation, the rabbits in the control (n = 7) and L-arginine (n = 7) group were sacrificed. BrdU 40 mg/kg (Sigma Chemical, St. Louis, MO) was administered intravenously one hour before sacrifice. The vein grafts were then exposed and isolated. After heparinization (200 IU/kg intravenously), the vein grafts were perfusion fixed in situ at 100 cm H₂O with 4% paraformaldehyde for 30 min. The perfused autologous vein graft, including anastomosis, was totally removed and immersed in the same fixative overnight at 4°C [20,24].

2.5. Sample processing and immunohistochemical staining

The middle portion of the harvested graft was used for histological studies. Each sample was paraffin-embedded and cut using a microtome into four micrometer thick sections, which were then mounted on glass slides. For the immunohistological analysis, monoclonal anti-rabbit macrophage, RAM-11 (Dako, Carpinteria, CA), was used [20]. Immunohistochemical staining was done as follows using the avidine–biotin complex (ABC) technique; the deparaffinized sections were treated with 0.3% hydrogen peroxidase.
rings were stimulated with 118 mM K⁺ at 17 Hz. During the 30 min equilibration period, the vessel contraction were stored in a Macintosh computer using a data acquisition system MacLab with a sampling rate of 8 kHz. Visualization of the peroxidase was achieved using the diaminobenzine (DAB) method. Each section was counterstained with hematoxylin–eosin and examined under a transmission light microscope [20].

2.6. BrdU staining and measurement of the labeling index

Each section was deparaffinized in a xylene–ethanol series. For DNA denaturation, the sections were first incubated in 2 M HCl for one hour and neutralized with 0.1 M sodium tetraborate, then digested with 0.05% protease (type XXV, Sigma Chemical, St. Louis, MO) for 5 min at 37°C, and rinsed with PBS. After the inhibition of endogenous peroxidase and incubation with normal rabbit serum, the sections were incubated with anti-BrdU monoclonal mouse antibody (1:50, Becton Dickinson Immunocytometry Systems, Mountain View, CA), overnight at 4°C. The sections were incubated with L-NMMA for 30 min. In the presence of L-NMMA, the endothelium-dependent responses to ACh (10⁻⁹–10⁻⁴ M) were examined.

For relaxations, the effective concentration of vasodilators causing 50% inhibition (ED₅₀) of the contractions to norepinephrine (3 × 10⁻⁷ M) was calculated from each concentration–response curve, and the means of these values were presented as the negative logarithm of the molar concentration.

The following drugs were used. acetylcholine chloride (ACh), 1-norepinephrine bicarbonate (NE) and L-NMMA (L-N⁵-monomethyl arginine) (all from Sigma Chemical, St. Louis, MO).

2.8. Assessment of intimal thickness

The sections were stained with hematoxylin–eosin and using the elastic van Gieson's method. Intimal thickness was measured with an ocular cytometer placed on the ocular lens of a light microscope at a magnification of 400x. The average intimal thickness of more than eight randomly selected points of each sample was taken as the degree of intimal thickness [26]. The area of the intima and media were also calculated by videomorphometry (Cosmowine IS; Nikon, Tokyo, Japan), and the ratio of the intimal and medial areas was also calculated (intima/media ratio = intimal area/medial area) [27].

2.9. Statistical analysis

Statistical evaluation of the data was made using Student's t-test for unpaired observations. When more than two means were compared, an analysis of variance was used. If the value was statistically significant, Scheffe's test for multiple comparisons was used to identify differences among the groups [28]. A value of p < 0.05 was considered to have statistical significance.

3. Results

All animals survived, and all the grafts were patent at harvest.
Table 1
Effects of 4-week L-arginine treatment on cholesterol-fed rabbits

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (n = 7)</th>
<th>Cholesterol/L-arginine (n = 7)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>2954 ± 172</td>
<td>2940 ± 180</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>The day of grafting</td>
<td>105 ± 11</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>Postoperative 4 weeks</td>
<td>103 ± 14</td>
<td>104 ± 13</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>35.3 ± 10.6</td>
<td>34.4 ± 3.8</td>
</tr>
<tr>
<td>Plasma arginine level (μmol/l)</td>
<td>185.5 ± 49.9</td>
<td>332.1 ± 150.7*</td>
</tr>
<tr>
<td>Plasma citrulline level (μmol/l)</td>
<td>110.1 ± 27.1</td>
<td>139.6 ± 37.0*</td>
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</table>

All values are the mean ± S.D.
* p < 0.05 compared with cholesterol group.

3.1. Systemic measurements

The animal body weights at the time of sacrifice were not different between the two groups. At operative day and 4 weeks after, the systolic arterial pressure was comparable in both the control and arginine groups. In addition, at the time of sacrifice, the plasma cholesterol levels were not significantly different between the two groups. However, those cholesterol levels were significantly higher than those of normolipidemic rabbits (n = 7; 1.1 ± 0.6 mmol/l). At the time of sacrifice, the plasma arginine levels and the citrulline levels in the arginine group were significantly higher than those in the control group (Table 1).

3.2. Endothelium-dependent responses

Precontracted vein grafts taken from control group (n = 4) did not relax to ACh, whereas those from the arginine group (n = 4), ACh caused endothelium-dependent relaxation in a dose dependent manner. Maximum relaxations

Fig. 1. Microscopic features of the mid-portion of autologous vein grafts of cholesterol-fed rabbits with (a and c) or without (b and d) L-arginine administration. (a,b) Rabbit vein grafts 2 weeks after implantation. The cross section of vein grafts in the arginine group seemed to be a reduced degree of intimal thickness compared with that of the control group, although the difference was not significant. (c,d) Rabbit vein grafts 4 weeks after implantation. The intimal thickness in the arginine group was significantly reduced compared with that of the control group. (Elastic Van Gieson’s stain, original magnification×200). The arrow indicates the thickened intima.
Table 2

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<th>Cholesterol (n = 5)</th>
<th>Cholesterol/l-arginine (n = 5)</th>
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<tr>
<td>Intimal thickness (μm)</td>
<td>67.7 ± 36.5</td>
<td>34.5 ± 19.0</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>1.13 ± 0.36</td>
<td>0.71 ± 0.27</td>
</tr>
<tr>
<td>BrdU labelling index</td>
<td>0.06 ± 0.02</td>
<td>0.03 ± 0.01*</td>
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All values are the mean ± S.D.

*p < 0.05 compared with cholesterol group.

were 55.3 ± 12.8% and ED₅₀ (−log M) was 5.3 ± 0.8. Preincubation with 10⁻⁴ M 1-NMMA completely abolished the endothelium-dependent relaxations (n = 3).

3.3. Progression of intimal thickness

At 2 weeks after operation, the vein grafts in the arginine group showed a reduced degree of intimal thickness compared with that of the control group, although the difference was not significant. The intima/media ratio was also comparable in both groups (Table 2 and Fig. 1).

At 4 weeks after operation, the vein grafts in both the arginine group and the control group showed a remarkable degree of intimal thickness development in the smooth muscle cells. However, the intimal thickness in the arginine group was significantly reduced compared with that of the control group. The intima/media ratio in the arginine group was also significantly inhibited, compared to that of the control group (Table 3 and Fig. 1).

Table 3

<table>
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<tr>
<th></th>
<th>Cholesterol</th>
<th>Cholesterol/l-arginine</th>
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<tbody>
<tr>
<td>Intimal hyperplasia (μm, n = 7)</td>
<td>164.2 ± 55.0</td>
<td>88.2 ± 34.5*</td>
</tr>
<tr>
<td>Intima/media ratio (n = 7)</td>
<td>1.40 ± 0.28</td>
<td>1.04 ± 0.17*</td>
</tr>
<tr>
<td>BrdU labelling index (n = 5)</td>
<td>0.04 ± 0.03</td>
<td>0.01 ± 0.02</td>
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All values are the mean ± S.D.

*p < 0.05 compared with cholesterol group.

3.4. Immunohistochemical distribution of the foamy macrophages

At 2 weeks after operation, the cross section of the grafts demonstrated thin fibrocellular neointima, and
RAM-11 positive foamy macrophages were scattered in the outer layer of the thickened intima in both groups (Fig. 2a and b). Four weeks after implantation, the autologous vein grafts of cholesterol-fed rabbits showed markedly thickened neo-intima diffusely containing many RAM-11 positive foam cells (Fig. 2d). In contrast, the vein grafts implanted under the hypercholesterolemia with L-arginine administration demonstrated reduced neo-intima lesion consisting mainly of smooth muscle cells. The RAM-11 positive foamy macrophages were observed scattered in the inner and outer layers of the thickened intima (Fig. 2c).

3.5. BrdU incorporation

The BrdU labeling index (LI) at 2 weeks after operation was significantly higher in the control group, compared with that in the arginine group (Table 2).

The number of BrdU positive cells decreased in both groups at 4 weeks after operation. The BrdU LI was slightly higher in the control group, compared with that in the arginine group, although the difference was not significant (Table 3).

4. Discussion

Late graft failure is usually attributed to graft thrombosis, based on either intimal thickness or a progression of the underlying atherosclerotic vascular disease [3,4]. The critical relationship between endothelial cells and the development of intimal thickness has been well recognized. Endothelial cells are known to release growth factors and heparin-like compounds, which likely have a major influence on surrounding cells and also help maintain normal vessel wall homeostasis [29–32]. Intimal thickness is the result of complex interactions among platelets, leukocytes and smooth muscle cells, all of which are affected by NO [33,34]. We and other investigators demonstrated a reduced production of NO in experimental autogenous vein grafts [21,28,30,35,36]. On the other hand, the enhanced EDRF production reduced the degree of intimal thickness in the vein grafts [37]. Thus, a relationship may therefore exist between NO and intimal thickness in vein grafts [38].

L-arginine is the precursor for the formation of NO [5]. The administration of L-arginine has been shown to reverse defects in NO activity and also reduce the development of proliferative atherosclerotic lesions [14]. Tarry et al. [15] demonstrated that L-arginine supplementation enhances NO production at the site of vascular healing and may reduce intimal thickness after balloon angioplasty. In addition, in the rabbit vein grafts, L-arginine reduced experimental vein graft intimal thickness [16]. The present results demonstrate that the elevation of plasma L-arginine and L-citrulline levels, which is a by-product of NO [39], may thus reflect the increase in NO production. These changes occurred despite the fact that L-arginine supplementation did not affect the serum cholesterol level. These data are similar to previous reports [15,16] in that L-arginine supplementation enhances the NO production in the endothelium and may thus inhibit the degree of intimal thickness in vein grafts in hypercholesterolemic animal models.

Hypercholesterolemia accelerates both atherosclerosis and graft failure in vein grafts [19]. Several studies thus demonstrated that NO-mediated relaxation is impaired in hypercholesterolemic animals [39,40] and humans [41]. This reduction of NO activity in associated hypercholesterolemia appears to be caused by a reduced synthesis and/or increased degradation of NO. On the other hand, Cooke et al. [14] showed that long-term L-arginine supplementation in hypercholesterolemic rabbits decreases atherogenesis in the aortic wall with decreased histologic lesion development, a reduced SMC proliferation, and a preservation of the endothelium-dependent relaxation. The results of the present study are similar, in that the treatment of the cholesterol-fed animals with arginine preserved ACh-induced endothelium-dependent relaxations. In addition, these endothelium-dependent relaxations were abolished by L-NMMA. These results demonstrated that the effects of L-arginine on the inhibition in the intimal thickness caused by hypercholesterolemic vein grafts are mediated through the enhancement of NO production.

In the presence of hyperlipidemia, endothelial dysfunction as a result of exposure to agents, such as oxidized low density lipoprotein (LDL) increases monocyte/macrophage adherence, and they migrate beneath the endothelium. The macrophages become large foam cells due to lipid accumulation, then form a fatty streak, the initial lesion of atherosclerosis [42]. Interestingly, the present results demonstrated that in the immunohistochemical distribution of macrophages, the RAM-11 positive foamy macrophages in the arginine group seemed to be reduced in number in the inner and outer layers of the thickened intima without changing the plasma cholesterol level. These results suggest that one of the mechanisms of NO inhibiting in the intimal hyperplasia may modulate monocyte requirement or foam cell lipid accumulation [27].

Zwolak et al. [43] showed that smooth muscle cell (SMC) proliferation in the thickened intima was maximal at one week after grafting. Similarly, Itoh et al. [20] also showed with BrdU incorporation that intimal SMC accelerated to two weeks after implantation, and that this acceleration was enhanced in rabbit femoral vein grafts if hypercholesterolemia was present. The present experiments demonstrated that the BrdU labeling index at 2 weeks after operation was significantly inhibited in the arginine group, compared with that of the control group, although after four weeks the BrdU labeling index was comparable between the two groups. On the other hand, at 2 weeks after operation, the degree of intimal thickness was comparable between the two groups, while at 4 weeks after operation, the degree of intimal hyperplasia in the arginine group significantly reduced compared with that of the control.
group. These results suggest that SMC proliferation of the jugular vein graft wall in hypercholesterolemic rabbits is rapid at the early stage after graft implantation and the proliferation of intimal cell in vein grafts was accelerated prior to the development of intimal thickness. NO is a potent inhibitor of SMC growth [11,44]. Therefore, L-arginine inhibited intimal SMC proliferation, thus inhibiting intimal thickness. To our knowledge, the present results demonstrate for the first time that L-arginine supplementation significantly inhibited smooth muscle cell proliferation of the vein grafts.

In conclusion, we demonstrated that the chronic administration of L-arginine, the precursor of NO, inhibited intimal thickness in jugular autogenous vein grafts in hypercholesterolemic rabbits by inhibiting the proliferation of SMC. The enhancement of NO production by the endothelium may thus be beneficial for preventing late graft failure.

Acknowledgements

We thank Mr. Brian Quinn for reading this manuscript.

References

[31] DiCorleto PE, Bowen-Pope DF. Cultured endothelial cells produce a...


