Enhanced Monocyte Tissue Factor Response After Experimental Balloon Angioplasty in Hypercholesterolemic Rabbit: Inhibition With Dietary L-Arginine

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Background—There is evidence that tissue factor (TF) is a major contributor to the thrombogenicity of a ruptured atherosclerotic plaque. Nitric oxide (NO) has antiatherogenic and antithrombotic properties. We investigated whether L-arginine (L-arg), the endogenous precursor of NO, might affect the ability of monocytes to produce TF.

Methods and Results—We studied TF expression in 18 rabbits with atherosclerosis induced by bilateral iliac damage and 10 weeks of a 2% cholesterol diet. Six weeks after the initiation of the diet, an angioplasty was performed. After angioplasty, the surviving rabbits (n=15) were randomized to receive L-arg (2.25%) supplementation in drinking water (L-arg group, n=8) or no treatment (untreated group, n=7). TF expression was evaluated in mononuclear cells from arterial blood in the presence and absence of endotoxin stimulation. Monocyte TF expression, as assessed with an amidolytic assay, did not differ significantly before or after the induction of atherosclerotic lesions (87±15 versus 70±12 mU of TF/1000 monocytes, P=NS). Endotoxin-stimulated TF activity increased significantly 4 weeks after angioplasty (138±22 versus 70±12 mU of TF/1000 monocytes, P=0.02). This increase was blunted by L-arg (43±16 mU of TF/1000 monocytes, P=0.01).

Conclusions—This study demonstrates that angioplasty-induced plaque rupture is associated with a marked increase in monocyte TF response that is blunted by the oral administration of L-arg. This suggests that the documented antithrombotic properties of NO may be related in part to an inhibitory effect on monocyte TF response. (Circulation. 1998;98:1776-1782.)

Key Words: tissue factor • monocytes • nitric oxide • atherosclerosis

Monocytes and macrophages are involved in the progression of atherosclerosis and in the pathogenesis of thrombosis.1,2 Monocytes can express tissue factor (TF),3,4 which is present in atheromatous plaques.5,6 There is evidence that TF is a major contributor to the thrombogenicity of ruptured plaques.7

The TF gene in monocytes is controlled by several transcription factors activated by external signals, such as growth factors, inflammatory cytokines (interleukin-1B and tumor necrosis factor-α), oxidized LDLs, and endotoxin. The induction of the TF gene in monocytes stimulated by endotoxin is mediated by the interaction of transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) with their corresponding binding sites that are present in the TF promoter region.8 The TF gene shares these regulatory mechanisms with other genes involved in leukocyte adhesion to endothelial cells, activated through a common oxidant-sensitive transcriptional pathway leading to the expression of endothelial proteins (eg, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and monocyte chemotactic protein-1).9,10

Nitric oxide (NO) plays an important role in vascular regulation through its vasodilatory,11 antiatherogenic,12 and antithrombotic properties. NO inhibits platelet adhesion and aggregation13 and modulates smooth muscle cell proliferation and migration.14 NO limits cytokine-induced endothelial activation15,16 and modulates the expression of monocyte chemotactic protein-1 in cultured endothelial cells17 through a decrease in NF-κB-binding activity.18 L-Arg decreases the adhesiveness of monocytes to the endothelium through inhibition of endothelium/leukocyte adhesion molecule transcription19; L-arg limits the progression of atherosclerosis,12,19 restores endothelium-dependent vasodilation,19 and limits intimal proliferation of vascular smooth muscle cells after angioplasty.20,21 We hypothesized that contact between atherosclerotic plaques and blood could increase TF expression by circulat-
ing monocytes and that NO might limit this response. To test these hypotheses, we used a rabbit model of induced atherosclerosis (bilateral iliac injury and an atherogenic diet), and we performed angioplasty when atherosclerotic lesions were established. In this model, we studied the effect of L-arg, the endogenous NO precursor, on TF expression by circulating monocytes.

**Methods**

Male New Zealand White rabbits (Charles River, Saint Aubin lès Elbronn, France) with an initial body weight of 3.0 to 3.5 kg were used for this study. All experiments were conducted in compliance with the position of the American Physiological Society on research animal use.

**Induction of Atherosclerosis**

Bilateral iliac atherosclerosis was induced according to the method described by Kakuta et al.23 Rabbits were anesthetized with ethyl carbamate (1 g/kg IV); after exposure of the femoral arteries, a 3F Fogarty balloon catheter was inserted to a distance of 20 cm, inflated until contact was made with the endothelium, and pulled back (3 times in each iliac artery). All animals were placed on a rabbit chow diet (200 g/d) containing 2% cholesterol. After 6 weeks, angiography was performed to confirm the existence of iliac lesions. Angioplasty allocation.

**Angioplasty**

Rabbits were anesthetized as described above. A 2.5-mm Bard coronary transluminal balloon angioplasty catheter was introduced via the carotid artery, and the balloon was positioned under fluoroscopy at the site of the iliac artery stenosis. Three successive 1-minute inflations at 6 atm were performed. After angioplasty, rabbits were again fed the cholesterol-supplemented diet and were randomized into 2 groups. The active treatment group (L-arg group, n = 8) received 2.25% L-arg hydrochloride (Sigma Chemical Co) in a limited quantity of drinking water (200 mL) every morning for 4 weeks. The untreated group (n = 7) received an equal quantity of plain drinking water each morning. When the animals had drunk this water, they were allowed free access to drinking water for the remainder of the day. The dose of L-arg (2.25%) was chosen based on the previous studies of Cooke et al.,23 who demonstrated that this dose was well tolerated and resulted in an increase in plasma free arginine levels compared with controls. Typical angiographic findings before and after angioplasty and a histological cross section of iliac artery after angioplasty are shown in Figure 1.

**Study Groups**

Blood samples were taken at 3 time points (before angioplasty) in all the rabbits to establish baseline characteristics for the entire population: at baseline (n = 18), at 3 weeks after iliac denudation and the initiation of a high cholesterol diet (n = 18), and at 6 weeks (just before angioplasty) in the surviving rabbits (n = 15) (Figure 2). Additional blood samples were taken 4 weeks after angioplasty in the L-arg–treated rabbits (n = 8) and in the untreated rabbits (n = 7).

**Blood Samples**

Blood samples were taken under sterile conditions from the ear artery. Three samples of blood were obtained from each rabbit: 5 mL was collected in lithium heparin (143 USP units) for mononuclear isolation, 2 mL was collected in sodium citrate (1:10, 3.8%) for plasma analyses, and 1 mL was collected in EDTA for hematomatological analyses (Coulter MAXM). The total white blood cell counts were verified manually. Peripheral blood smears for the differential white cell counts were stained with May-Grünwald-Giemsa. Each count was performed by 3 investigators, who were blinded to treatment allocation.

Blood samples collected in lithium heparin and in EDTA were diluted to decrease measurement artifacts due to severely lipemic blood.

**Isolation of Mononuclear Cells and Cell Culture**

The mononuclear cells were isolated by gradient centrifugation (MSL, density = 1.077 ± 0.001, Eurobio), washed 2 times, and resuspended in RPMI 1640 (GIBCO) (3 × 10^6 cells/mL). Cell viability was >98% (trypan blue test). Monocytes composed 12 ± 1% (mean ± SEM) of the cells.

All reagents and culture supplies used in the study were free of endotoxin (chromogenic limulus amebocyte lysate [LAL] assay sensitivity, 0.025 endotoxin unit [EU]/mL). An aliquot of the freshly isolated mononuclear cells, referred to as noncultured cells, was frozen at −80°C. Other aliquots of cell preparations (3 × 10^6 cells/mL) suspended in RPMI 1640 without fetal calf serum were cultured for 16 hours at 37°C in a humidified 5% CO2 atmosphere, without or with stimulation by endotoxin at 5000 EU/mL (Escherichia coli 055:B5, Sigma Chemical); these are referred to as unstimulated and stimulated cells, respectively. At the end of the incubation period, monocytes were reisolated and frozen at −80°C.

**TF Activity Assay**

The frozen cells were lysed by the addition of 0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Triton X-100, and 0.1% BSA (60 μL/mL) for 30 minutes at 37°C with serial vortex mixing. TF activity was determined with a modified amidolytic assay.24 Briefly, lysed cell suspensions (50 μL) were incubated at 37°C in a microtiter plate (2 minutes) and mixed with 0.25 M CaCl2 (50 μL) (3-minute incubation) and prothrombin concentrate complex (Laboratoire de Fractionnement et des Biotechnologies) as a source of factor VII (50 μL, 3 U/mL). After the addition of 50 μL of the chromogenic substrate S2765 (Biogenic), the change in optical density at 410 nm was quantified with a microplate reader and converted to units of TF activity from log-log plots of serial dilutions of a rabbit brain thromboplastin (Cl; Stago). Arbitrarily, 1 mL of thromboplastin was assigned a value of 1000 U/mL TF. Results were expressed as mlU/mL TF/mL of blood.

The amidolytic activity was characterized as TF according to a neutralization procedure using mouse monoclonal antibody anti-rabbit–TF (AP-1); diluted (1:18) antibody (25 μL) was incubated with diluted TF standard or lysed cell suspensions for 30 minutes at 37°C. Then, the mixture was tested for amidolytic activity.

**Immunocytochemical Staining**

Immunocytochemical staining was performed on cytocentrifuged preparations with the use of AP-1 or mouse negative control (DAKO) and alkaline phosphatase anti-alkaline phosphatase complex (APAAP Kit system; DAKO).

Cells were fixed in buffered acetone/acetone/paraformaldehyde 4%. Mouse isotype antibody was used as a negative control. Anti-TF labeling is seen as a bright red color in the cytoplasm, with membrane reinforcement in the most positive cells (Figure 3).

**Assays on Plasma Samples**

Plasma samples were diluted to decrease measurement artifacts due to severely lipemic plasma.

Serum total cholesterol and triglyceride (TG) levels were determined with enzymatic assays using cholesterol esterase plus cholesterol oxidase and glycerol-3-phosphate oxidase, respectively (Biomerieux). The L-arg level was determined after deproteinization with 10% sulfosalicylic acid and was analyzed for free arginine (LC 300; Biotronic Instrument). Prothrombin time was measured by use of an automated clotting assay with calcified thromboplastin (Biomerieux). Fibrinogen levels were measured according to the Clauss technique (Biomerieux). Factors II, V, and VII–X levels were determined by an automated clotting assay (STA; Stago) with the use of calcified rabbit brain thromboplastin and human factor–deficient plasma (Stago).
Statistical Analysis

Results are expressed as mean±SEM. Data were analyzed using a nonparametric test (Kruskal-Wallis) to determine significant differences ($P<0.05$) in mean values between groups, followed by the Mann-Whitney $U$ test to test the significance of differences between groups.

Results

Blood Lipid and $\mathbf{L}$-Arg Levels

Serum cholesterol and TG levels were significantly higher in animals after 6 weeks on a high cholesterol diet (6 weeks: cholesterol, $3052±278$ mg/dL; TG, $489±147$ mg/dL; base-
There was no difference between the L-arg group and the untreated group for any of these parameters.

**Monocyte TF Activity**

Amidolytic activity was detectable in mononuclear cells after a 16-hour culture. Neutralization assay with TF antibody confirmed that the amidolytic activity was TF in all cases.

**Early Effects of Hypercholesterolemia and Iliac Denudation**

In unstimulated cells, a decrease in TF activity was observed 3 weeks after bilateral iliac injury and initiation of the atherogenic diet (20±3 versus 66±20 mU of TF/1000 monocytes, P=0.02) and remained lower than the baseline value at all subsequent time points (Figure 4A).

In stimulated cells, a significant decrease in monocyte TF activity was observed at 3 weeks (30±6 versus 87±15 mU of TF/1000 monocytes, P<0.005), followed by a trend toward normalization after 6 weeks (70±12 mU of TF/1000 monocytes, P<0.005 versus 3 weeks) (Figure 4A).

The results were similar when TF monocyte content was expressed per milliliter of blood (Figure 4B).

**Effects of Angioplasty With and Without L-Arg Supplementation**

In unstimulated cells, no significant difference in TF activity was observed between the group that received L-arg and the untreated group; TF activity was lower in rabbits with L-arg, but the difference was not statistically significant (Figure 5A).

In stimulated cells, TF activity was significantly greater 4 weeks after angioplasty in the untreated group compared with the value observed just before angioplasty (138±22 versus 70±12 mU of TF/1000 monocytes, P=0.02). This increase in stimulated TF activity was significantly less in the L-arg group than in the untreated group (43±16 versus 138±22 mU of TF/1000 monocytes, P=0.01) (Figure 5A).

**Figure 3.** Representative example of monocytes with (A) and without (B) qualitative evidence of TF expression. Magnification, ×1250.
The results were similar when TF monocyte content was expressed per milliliter of blood (Figure 5B).

**Immunocytochemistry**

The results of immunocytochemical staining with anti-TF antibody in stimulated monocytes were concordant with the results of the functional TF assays. At baseline, 71% of stimulated monocytes were TF positive; 63% were TF positive at 3 weeks, and 81% were positive at 6 weeks. At 4 weeks after angioplasty, 92% were TF positive in untreated animals versus 65% in the L-arg group.

**Changes in Other Parameters**

After 3 weeks on a high cholesterol diet, there was a significant increase in factor II (149±7% versus 94±5% at baseline, *P<0.0001) and factor VII+X (151±7% versus 92±4% at baseline, *P<0.0001), which remained significant at all the subsequent time points studied. No significant difference in factor V levels was observed (115±8% versus 103±10% at baseline, *P=NS). Fibrinogen levels were significantly lower after 6 weeks in animals receiving a high cholesterol diet compared with baseline (2.9±0.3 versus 4.6±0.4 g/L, *P=0.0004), and levels remained at this level 4 weeks after angioplasty (2.5±0.3 g/L).

There was no difference between the L-arg group and the untreated group with respect to the levels of plasma coagulation factors (Table 2).

**Discussion**

The major finding of this study was the demonstration that the performance of angioplasty in a rabbit model of atherosclerosis induced by a high cholesterol diet and iliac denudation is associated with a significant increase in monocyte TF response, which is blunted by dietary L-arg supplementation. This is the first study to investigate monocyte TF responses in vivo in a rabbit model of induced atherosclerosis and to study the effect of the NO precursor L-arg in this model.

**Effect of Endothelial Denudation and Hypercholesterolemia**

We observed a decrease in monocyte TF activity in the weeks after initiation of a high cholesterol diet associated with bilateral iliac injury. The reasons for this decrease are unclear, but they could be related to the disappearance from the bloodstream of the most active monocytes; this hypothesis is supported by the observation that the monocyte count tended to decrease over the same period. This may reflect an increase in monocyte adhesion and penetration into the vascular wall.1,25 Alternatively, hypercholesterolemia could modify the lipidic composition of cell membranes, modulating TF activity, or it increase the specific TF pathway inhibitor, as previously shown in humans.26

Additional significant changes in the levels of coagulation factors and platelets were observed. A decrease in the platelet count and the levels of fibrinogen occurred, suggesting an associated consumption process. Vitamin K–dependent factors (factors VII and X) increased significantly, as reported previously.27,28 This increase has been shown to reflect an increase in the rate of synthesis, or activation.

**Effects of Angioplasty**

At 4 weeks after angioplasty, we observed an increased monocyte TF response to endotoxin. This probably reflects an increase in the degree of activation of circulating monocytes.

### Table 1. Effect of Hypercholesterolemia, Angioplasty, and L-Arg on Total White Blood Cell, Monocyte, and Platelet Counts

<table>
<thead>
<tr>
<th></th>
<th>Total White Blood Cells, 10⁹/L</th>
<th>Monocytes, 10⁹/L</th>
<th>Platelets, 10⁹/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>7.4±0.5</td>
<td>0.23±0.04</td>
<td>464±28</td>
</tr>
<tr>
<td>3 wk</td>
<td>7.5±0.4</td>
<td>0.15±0.02</td>
<td>373±22</td>
</tr>
<tr>
<td>6 wks, before angioplasty</td>
<td>10.1±0.6†</td>
<td>0.22±0.05</td>
<td>321±29†</td>
</tr>
</tbody>
</table>

**Values are mean±SEM.**

*P<0.05 vs baseline, †P<0.005 vs baseline.
as already described in human coronary disease, specifically in unstable coronary syndromes. Leukocyte activation has been described in the early days after coronary angioplasty in humans, but no previous study has investigated leukocyte activation several weeks after balloon injury. A potential hypothesis is that the rupture of lipid-rich atheromatous plaque by angioplasty allows direct contact between the plaque components, including foam cells, activated T cells, and activated macrophages, which could produce inflammatory cytokines and activate the TF gene. This hypothesis is supported by the observation that animals that received a high cholesterol diet over an equivalent time period but were not subjected to iliac denudation did not have demonstrable changes in levels of TF response (data not shown), as previously reported.

**Effect of l-Arg**

Dietary l-arg supplementation had a significant effect on monocyte TF response. The increase in stimulated monocyte TF response that occurred in untreated animals was significantly blunted by oral administration of l-arg. The mechanisms of the inhibitory effect of l-arg on monocyte TF activity are unclear. This effect could be related to the antiatherogenic effect of l-arg: it has been demonstrated that hypercholesterolemia increases the generation of superoxide anion in endothelial cells, which plays a key role in the pathogenesis of atherosclerosis, and this process is limited by l-arg supplementation.

NO can also inhibit the transcriptional protein NF-κB or scavenge the superoxide anion that activates NF-κB. Because TF gene activation by endotoxin is mediated by NF-κB activation, the modulation by NO of monocyte TF response in our model could occur through limitation of this common oxidant-sensitive transcriptional pathway. Indeed, recent data indicate that an antioxidant phytoelement can inhibit cytokine-induced TF gene expression in part through inhibition of NF-κB activation in cultured endothelial cells. To our knowledge, the effect of NO on monocyte TF response had not previously been studied. Alternatively, Brisseau et al demonstrated in vitro that antioxidants decreased TF expression by monocytes/macrophages through a post-transcriptional effect. These elements combined suggest that NO may reduce monocyte TF response through direct transcriptional or post-transcriptional mechanisms. Monocyte TF mRNA determination will provide further insights into the relative contribution of these two possible mechanisms.

**Study Limitations**

The extrapolation of data from any animal model to human atherosclerosis requires caution. However, the atherosclerotic rabbit model we used has been demonstrated to have several features in common with human atherosclerosis, and its reproducibility has been demonstrated in several previous studies. A diet supplemented with 2% cholesterol results in very high cholesterol levels in this model and severe atherosclerotic lesions and is associated with significant morbidity rates in the animals. The results of the present study might differ in animals with lesser degrees of cholesterol supplementation. Similarly, stimulation with endotoxin may not reflect the pathophysiological changes seen in atherosclerosis; however, it is a powerful stimulator of TF gene, and infectious agents are possible aggravating factors in the evolution of atherosclerosis.

In summary, exogenous administration of l-arg, the NO precursor, blunts the increase of TF response in stimulated monocytes after angioplasty in a rabbit model of induced atherosclerosis. These results suggest that the antithrombotic properties of NO could also be related to an inhibitory effect on monocyte TF response, in addition to its known effects on endothelial and platelet functions. Local generation of sufficiently high amounts of NO by endothelial cells or macrophages, through oral l-arg supplementation, may induce a reduction in TF-mediated hemostatic activation in complicated atherosclerosis. L-Arg is known to restore endothelium-dependent dilation and to inhibit platelet aggregation in humans. As a therapeutic agent, l-arg may have a potential clinical impact via these antithrombotic properties in the treatment or the prevention of human vascular diseases, including atherosclerosis, thrombosis, and septic shock. A complete understanding of the physiological and pathophysiological roles of l-arg must await further studies.

**TABLE 2. Factors II, V, and VII Plus X and Fibrinogen Levels in Untreated and l-Arg Groups**

<table>
<thead>
<tr>
<th></th>
<th>Untreated Group (n=7)</th>
<th>l-Arg Group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II, %</td>
<td>151±12</td>
<td>177±15</td>
</tr>
<tr>
<td>Factor V, %</td>
<td>76±11</td>
<td>106±13</td>
</tr>
<tr>
<td>Factor VII plus X, %</td>
<td>153±7</td>
<td>187±16</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>2.4±0.3</td>
<td>2.4±0.2</td>
</tr>
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Values are mean±SEM.
Acknowledgments
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References
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