Basic Fibroblast Growth Factor Increases Tissue Factor Expression in Circulating Monocytes and in Vascular Wall

Delphine Corseaux, PhD; Thibaud Meurice, MD; Isabelle Six, PhD; Lucia Rugeri, MD; Michael D. Ezekowitz, MD, PhD; Philippe Rouvier, MD; Régis Bordet, MD; Christophe Bauters, MD; Brigitte Jude, MD

Background—Basic fibroblast growth factor (bFGF) promotes vascular repair and angiogenesis and can induce in vitro tissue factor (TF), a potent agent initiating thrombogenesis, which probably plays a role in angiogenesis. We investigated whether bFGF administration induced TF expression by monocytes and vascular cells.

Methods and Results—We studied TF expression in normally fed (n=16) and cholesterol-fed (2% for 6 weeks, n=16) rabbits. Animals were then randomized to receive intravenous bFGF (2.5 μg twice weekly for 3 weeks) or saline injections. TF expression was evaluated in mononuclear cells from arterial blood and in aortic sections by an immunohistochemical assay using a monoclonal anti-rabbit TF antibody (activator protein 1). Monocyte TF expression was increased by bFGF administration in both normal and hypercholesterolemic rabbits (129±45 versus 19±3 mU TF/1000 monocytes, P<0.05, and 31±12 versus 7±1 mU TF/1000 monocytes, P<0.005, respectively) and was further increased by stimulation of monocytes by endotoxin in vitro. TF expression was lower in hypercholesterolemic rabbits than in normal rabbits. In the media of the vascular wall, bFGF induced strong TF expression in normal rabbits and only weak TF expression in hypercholesterolemic ones.

Conclusions—This study demonstrates that systemic administration of bFGF induces an impressive increase of TF expression in circulating monocytes and in the vascular wall in normal and to a lower extent in hypercholesterolemic rabbits. The significance of this observation in terms of inducing thrombosis in vivo needs clarification. (Circulation. 2000;101:2000-2006.)

Key Words: growth substances ■ leukocytes ■ vessels

Growth factors induce the proliferation and migration of endothelial cells and play a role in angiogenesis and in the restoration of endothelial integrity after trauma.1 Of particular interest, basic fibroblast growth factor (bFGF) can stimulate proliferation, morphological changes, and migration of vascular smooth muscle cells (SMCs) and vascular endothelial cells in vitro; in vivo, bFGF has been shown to promote reendothelialization with functional endothelium after balloon injury or induced atherosclerosis.2-5 Although these findings are advantageous, numerous genes can be activated by growth factors, including bFGF, that may produce undesired effects. For instance, tissue factor (TF) probably plays an important role in both thrombogenesis6 and angiogenesis.7-9 The regulation of the TF gene is controlled by several transcription factors activated by inflammatory cytokines (interleukin-1β and tumor necrosis factor-α), oxidized LDLs, and endotoxin.10,11 In vitro growth factors, such as platelet-derived growth factor, FGF, transforming growth factor-β, or epidermal growth factor, can induce TF expression in fibroblasts and SMCs.12-15

To investigate whether bFGF administration upregulates TF expression in the vasculature, we evaluated TF expression in monocytes and in the vascular wall in normal and hypercholesterolemic rabbits treated with bFGF.

Methods

Study Protocol
A total of 32 male New Zealand White rabbits (3 to 3.5 kg, Charles River France) were studied. Sixteen animals were fed a standard rabbit diet and randomized to a 3-week treatment with either bFGF (bFGF group, n=8) or placebo (control group, n=8). Plasma cholesterol levels were 32±2 mg/dL. Sixteen other rabbits were placed on a 200-g/d rabbit chow diet containing 2% cholesterol for 6 weeks; plasma cholesterol increased to 1950±354 mg/dL (P<0.0001). Animals were then transferred to a standard rabbit diet and were randomized to a 3-week treatment with either bFGF (hypercholesterolemic bFGF group, n=8) or placebo (hypercholesterolemic control group, n=8). At the end of the 3-week treatment period, plasma cholesterol had decreased to 908±183 mg/dL in the hypercholesterolemic bFGF group and to 1053±122 mg/dL in the hypercholesterolemic control group.
In the normocholesterolemic and the hypercholesterolemic rabbits assigned to bFGF treatment, human recombinant bFGF boluses (2.5 μg bFGF in 1 mL 0.5% albumin per IV injection, Sigma) were administered twice weekly during a 3-week period. The rabbits assigned to placebo treatment received vehicle injections of albumin twice weekly.

Blood was obtained 3 days after the last injection of bFGF or placebo. All animals were then euthanized with sodium pentobarbital for histological studies. All experiments were conducted in compliance with the position of the American Physiological Society on research animal use.

**Blood Samples**

Blood was sampled under sterile conditions from the ear artery: 5 mL in sodium citrate (1:10, 3.8%) for coagulation and lipid analysis, and 1 mL in EDTA for blood cell counts.

Samples collected on sodium citrate were centrifuged (1500 g, 1 hour) and separated. The plasma was frozen (−80°C) for further analysis.

Samples collected on EDTA were used for hematological analyses with a Coulter MAXIM. The white blood cell counts were verified manually. Peripheral blood smears for differential white cell counts were stained with May–Grünwald–Giemsa stain and examined by 3 investigators who were blinded to the treatment allocation.

**Mononuclear Cell Culture**

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

All reagents and culture supplies used were free of endotoxin (chromogenic limulus amoebocyte lysate 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.

Aliquots of cell preparations (3×10^6 cells/mL) suspended in RPMI 1640 without FCS were cultured for 16 hours at 37°C in a humidified 5% CO₂ atmosphere, without or with stimulation by endotoxin (5000 EU/mL, *Escherichia coli* 055:B5, Sigma); these are referred to as unstimulated and stimulated cells, respectively. At the end of the incubation period, cells were resuspended and frozen at −80°C.

**TF Activity Assay**

The frozen cells were lysed by addition of 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 0.1% Triton X100, 0.1% BSA (60 μg/mL) for 30 minutes at 37°C associated with serial vortex mixing. TF activity was determined by a modified amidolytic assay as previously described.15 Briefly, lysed cell suspensions (50 μL) were incubated at 37°C mixed with CaCl₂ 0.25 mol/L (50 μL) and prothrombin concentrate complex (Laboratoire de Fractionnement et des Biotechnologies) as a source of factor VII (50 μL, 3 IU/mL). After addition of 50 μL of the chromogenic substrate S2765 (Biogenic), the change in optical density (410 nm) was quantified and converted to units of TF activity from serial dilutions of a rabbit brain thromboplastin (Cl+, Stago). One milliliter of thromboplastin was assigned a value of 1000 U/mL of TF. Results were expressed as mU/1000 monocytes.

The procoagulant activity was characterized as TF by a neutralization procedure using mouse monoclonal antibody anti-rabbit TF (AP-1, gift from M.D. Ezekowitz, Yale University, New Haven, Conn); 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 procoagulant activity. The percentage neutralization was calculated with the rabbit brain thromboplastin without and with antibody used as 0% and 100%, respectively.

**Additional Assays on Plasma Samples**

Prothrombin time and fibrinogen levels were measured by clotting assays (Biomerieux).

Factor II, V, and VII+X levels were determined by an automated clotting assay (STA, Stago) using calcified rabbit thromboplastin and human factor–deficient plasma (Stago). Coagulation times were then expressed as percentages by comparison to standard curves constructed with serial dilutions of standard rabbit plasma in Owren Koller buffer (1/10 to 1/40 for factor II and factor VII+X and 1/100 to 1/400 for factor V).

**Histological Studies**

The abdominal aortas of all animals were dissected and cleaned by brief immersion in PBS, fixed in 4% paraformaldehyde for 10 minutes, and then maintained in 30% sucrose/distilled water for 4 hours. Tissues were embedded in OCT (methyl methacrylate) compounds, quick-frozen in isopentane, and stored at −80°C. Sections (6 μm) of the frozen tissues mounted on glass slides (Superfrost Plus) were immunohistochemically labeled with a murine monoclonal antibody against rabbit TF (AP-1). Briefly, frozen sections were air-dried for 1 hour, then blocked with 10% horse serum for 10 minutes at room temperature. TF antibody was diluted at 1/200 in PBS and incubated for 1 hour at 37°C. In parallel, a negative control without primary antibody was performed. Samples were washed 3 times with PBS and incubated with a biotinylated anti-mouse IgG ( Vectastain ABC kit, Vector Laboratories) diluted in PBS for 1 hour at room temperature. Samples were washed 3 times with PBS, incubated with ABC reagent for 1 hour, and color-developed for peroxidase with diaminobenzidine (12 μL H₂O₂, 15 mL PBS) for 3 minutes. Samples were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted with Eukitt (Pertex, Histolab).

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**TABLE 1. Total White Blood Cell and Monocyte Counts in Control, bFGF, Hypercholesterolemic Control, and Hypercholesterolemic bFGF Rabbits**

<table>
<thead>
<tr>
<th></th>
<th>Total White Blood Cells, 10^9/L</th>
<th>Monocytes, 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group, n=8</td>
<td>8.4±0.5</td>
<td>0.37±0.07</td>
</tr>
<tr>
<td>bFGF group, n=8</td>
<td>10.5±2.7</td>
<td>0.29±0.12</td>
</tr>
<tr>
<td>Hypercholesterolemic control group, n=8</td>
<td>9.2±0.8</td>
<td>0.27±0.07</td>
</tr>
<tr>
<td>Hypercholesterolemic group, n=8</td>
<td>10.4±1.1</td>
<td>0.59±0.12*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs hypercholesterolemic control group; $P<0.05 vs bFGF group.
independent investigators who were blinded to the treatment allocation assigned semiquantitative scores to the slides (0 corresponding to no TF staining, and 3 to strong TF staining, corresponding to adventitia staining).

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

Results

Total White Blood Cell and Monocyte Counts
The total white blood cell and monocyte counts are presented in Table 1. There was no significant difference in the total white blood cell count among all groups. The monocyte count in the hypercholesterolemic bFGF group was significantly higher than in the other groups.

Table: Factor II, V, VII+X, and Fibrinogen Levels and Platelet Count in Control, bFGF, Hypercholesterolemic Control, and Hypercholesterolemic bFGF Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Factor II, %</th>
<th>Factor V, %</th>
<th>Factor VII+X, %</th>
<th>Fibrinogen, g/L</th>
<th>Platelets, 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group, $n=8$</td>
<td>97 ± 3</td>
<td>88 ± 3</td>
<td>101 ± 4</td>
<td>3.4 ± 0.2</td>
<td>302 ± 26</td>
</tr>
<tr>
<td>bFGF group, $n=8$</td>
<td>91 ± 2</td>
<td>117 ± 11</td>
<td>88 ± 3$^*$</td>
<td>5.1 ± 0.6</td>
<td>420 ± 46$^*$</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>144 ± 9$^*$</td>
<td>121 ± 10$^*$</td>
<td>142 ± 7$^*$</td>
<td>2.7 ± 0.2$^*$</td>
<td>550 ± 61$^*$</td>
</tr>
<tr>
<td>control group, $n=8$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>129 ± 11$^*$</td>
<td>136 ± 13$^*$</td>
<td>143 ± 12$^*$</td>
<td>2.4 ± 0.3$^*$</td>
<td>576 ± 80$^*$</td>
</tr>
<tr>
<td>bFGF group, $n=8$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *$P<0.002$ vs control group; **$P<0.05$ vs control group; §$P<0.01$ vs bFGF group.

Effect of bFGF Administration in Normal Rabbits
TF activity was higher in the bFGF group than in the control group. This difference was significant in unstimulated cells ($31 ± 12$ versus $7 ± 1$ mU TF/1000 monocytes, $P<0.005$) and in stimulated cells ($185 ± 26$ versus $53 ± 12$ mU TF/1000 monocytes, $P<0.005$). However, this increase was not as high as it was in the normocholesterolemic rabbits treated with bFGF ($31 ± 12$ versus $129 ± 45$ mU TF/1000 monocytes, $P<0.05$).

Effect of Hypercholesterolemia
TF activity in unstimulated cells was significantly lower in the hypercholesterolemic control group than in the control group ($7 ± 1$ versus $19 ± 3$ mU/1000 monocytes, $P<0.05$). TF activity in stimulated cells was the same in the 2 groups ($53 ± 12$ versus $50 ± 11$ mU/1000 monocytes, $P=NS$).

Discussion
This is the first in vivo study to investigate the relationship of bFGF and TF expression on circulating monocytes and in the vessel wall. The major finding of this study is the demon-

Histological Studies
Abdominal vessels were studied by immunohistochemistry to map the TF distribution in the vessel wall. Representative photomicrographs are shown in Figure 2.

TF positivity was found predominantly in the adventitia in all groups. There was no TF expression in the intima or the media of control rabbits (Figure 2a). In rabbits treated with bFGF, TF was found in the intima and in the media. Endothelial staining was particularly evident (Figure 2b). In hypercholesterolemic control rabbits, minimal amounts of TF were detected in the media but none in the intima (Figure 2c). After bFGF treatment in hypercholesterolemic rabbits, a moderate increase of TF expression was seen in the media but not in the intima (Figure 2d). It is noteworthy that induction of TF expression by bFGF was not as high in cholesterol-fed rabbits as in normally fed rabbits (Figure 2d and 2b).

Changes in Other Parameters
In normal rabbits (Table 2), bFGF induced a decrease of the factor VII+X level and an increase of the fibrinogen level and platelet count. In the hypercholesterolemic groups, however, factor II, V, and VII+X levels and platelet counts increased and fibrinogen levels decreased with no further effect of bFGF.

Figure 2. Photomicrographs show localization of TF in control (a), bFGF (b), hypercholesterolemic control (c), and hypercholesterolemic bFGF (d) rabbit abdominal artery. TF was localized in the adventitia (A) of all groups, in the media (M) and intima (I) of bFGF rabbits (b), and in the media of hypercholesterolemic control and hypercholesterolemic bFGF rabbits (d). Note that in normocholesterolemic bFGF rabbits, immunohistochemical detection of TF is more intensive than in hypercholesterolemic bFGF rabbits. Magnification X 32.
stration that the administration of bFGF induced a significant increase in TF response in circulating monocytes and in the vascular wall. However, the effect of bFGF appears to be different when administered to normally fed rather than cholesterol-fed rabbits.

**Effect of bFGF Administration in Normal Rabbits**

The induction of TF gene by growth factors has already been demonstrated in fibroblasts and SMCs. Our results indicate that bFGF induces TF expression in vivo in monocytes and in vascular cells. Four types of FGF receptors have been identified; these are present in either monocytes/macrophages, endothelial cells, or SMCs.17–19 The binding of bFGF to its specific receptor triggers a cascade of events leading to signal transduction by extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase, Ras, and protein kinase C (PKC) pathways.20,21 Little is known about the induction mechanisms of TF expression by bFGF. However, a recent study has demonstrated that platelet-derived growth factor induces TF expression in human SMCs by an ERK pathway–dependent mechanism and, in part, by Ras and PKC pathway–dependent mechanisms.12 It is possible that bFGF could act in the same manner on TF expression in circulating monocytes and in cells of the vascular wall.

In bFGF-treated rabbits, stimulation of monocytes by endotoxin in vitro failed to induce a further increase of TF activity. Endotoxin is known to induce TF expression by a PKC pathway–dependent mechanism22 leading to AP-1 and nuclear factor-κB transcription factor activation. Because this pathway is also stimulated by growth factors and serum,23 it seems possible that the preliminary effect of bFGF has saturated the endotoxin activation pathway at the second messenger or at the transcription factor level. Interestingly, Pendurthi et al.24 recently demonstrated that bFGF pretreatment of endothelial cells prevents TF expression in response to phorbol myristate acetate. Taken together, these results indicate that the cellular effects of bFGF are different according to the stimulation context.

**Effect of Hypercholesterolemia**

In hypercholesterolemic rabbits, TF staining was observed in the media (not at all in the intima), indicating that TF induction was present in this group. By contrast, in circulating monocytes, TF response was lower than in the control group. This confirms data reported by our group16 and others.25 The reasons for this lower TF activation are unclear. The effects of lipids on monocyte TF response are complex. Monocyte TF response is decreased by LDL and lysophosphatidylcholine26,27 but is increased by oxidized LDL and free cholesterol.28,29 Moreover, hypercholesterolemia induces monocyte adhesion, penetration, and transformation into foam cells in the vascular wall. Because we observed that circulating monocytes decreased in hypercholesterolemic rabbits, it seems possible that these monocytes in hypercholesterolemic rabbits are a subset of the total monocyte population with impaired capacity to respond to stimulation.

**Effect of bFGF Administration in Hypercholesterolemic Rabbits**

In hypercholesterolemic rabbits, bFGF induced an increase of TF expression in circulating monocytes and in the vessel wall. In unstimulated monocytes, bFGF restored TF activity identically in the hypercholesterolemic and the control groups. However, TF induction in unstimulated monocytes by bFGF remained less important than in normocholesterolemic rabbits. By contrast, in hypercholesterolemic rabbits treated with bFGF, endotoxin induced a striking TF response, indicating a “priming” effect of bFGF. In the vascular wall of hypercholesterolemic rabbits, bFGF induced TF expression only in the media; no TF detection was observed in the intima. The effect of bFGF was less important than in the normocholesterolemic rabbits.

The different effects of bFGF in hypercholesterolemic and control rabbits are unclear. Hypercholesterolemia may interfere with the relationship between bFGF and its membrane receptors. Physiologically, bFGF is stored in the extracellular matrix30 and is associated with cell surface membranes31 by heparan sulfate proteoglycan (HSPG) binding. Moreover, HSPGs are necessary for the bFGF binding to its receptors to exert both autocrine and paracrine effects.32 But atherosclerosis involves a decrease of HSPG.33 Thus, hypercholesterolemia could decrease the affinity and/or the binding of bFGF to its receptors. Conversely, hypercholesterolemia may modulate the expression of the different bFGF receptors.19,34 It is noteworthy that in this model, hypercholesterolemia did not impair the monocyte TF response to endotoxin.

**Possible Consequences of Induction of TF by bFGF**

TF is known to be a potent activator of blood coagulation. The exposure of TF to blood and factor VII and its activation could lead to thrombosis mediated by bFGF treatment, shown particularly in the normal animals. Although this is theoretically possible, we found no evidence of coagulation factor consumption or decreased platelet count 3 days after the last bFGF injection. Moreover, no evidence of arterial thrombosis was observed.

Several previous studies indicate that TF is probably an important angiogenesis factor through mechanisms that have been discussed. TF regulates angiogenic properties of tumor cells in mice by increasing angiogenic factor (vascular endothelial growth factor, VEGF) and decreasing antiangiogenic factor (thrombospondin) production.7 TF is also involved in embryogenesis8,9 and promotes metastasis of melanoma cells.35 It was recently demonstrated that the TF–factor VIIa complex can also induce VEGF production.36 A cholesterol-rich diet in rabbits induces partial denudation of the endothelium and loss of functional properties. In our model, it was previously demonstrated that bFGF induces rapid reendothelialization, restores endothelial functions, and plays a role in angiogenesis.5 Little is known about the interaction of bFGF and TF in these processes. Importantly, it has been demonstrated in another model of vascular balloon injury that vascular cells can release both endogenous bFGF37 and TF.38

**Study Limitations**

The extrapolation of data from any animal model to humans requires caution. A diet supplemented with 2% cholesterol results in very high cholesterol levels and is associated with a rapid progression of the vascular lesions. This hypercho-
hemostatic rabbit model has been demonstrated to have features in common with humans, and its reproducibility has been demonstrated in several previous studies. Our results indicate that the particular dose regimen of bFGF used in this study induces TF expression in circulating monocytes and in the vascular wall in normal and in hypercholesterolemic rabbits. TF can increase the thrombogenicity of the vascular wall, and this effect can have important clinical implications. However, no evidence of consumption coagulopathy was found. Conversely, TF is probably an important angiogenic factor and can supplement the beneficial effects of angiogenic growth factor therapy. Further studies are necessary to understand the role of bFGF-induced TF expression and the therapeutic effect of bFGF.

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References

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