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

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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 thrombogenesis and angiogenesis.⁶⁻⁹ 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 lithium heparin (143 USP units) for mononuclear cell isolation, 2 mL in sodium citrate (1:10, 3.8%) for coagulation and lipid analysis, and 1 mL in EDTA for blood cell counts. Blood was centrifuged (1500 g, 2 minutes, +4°C) and separated. The plasma was frozen (−80°C) for further analysis.

**Mononuclear Cell Culture**

The mononuclear cells were isolated by gradient centrifugation (MSL, 80°C). Aliquots 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 (Gibco) (3% 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 were 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.19 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.

**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>Hypercholesterolmic bFGF 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.
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 ×32.

Table 2. Factor II, V, VII+X, and Fibrinogen Levels and Platelet Count in Control, bFGF, Hypercholesterolemic Control, and Hypercholesterolemic bFGF Rabbits

<table>
<thead>
<tr>
<th>Condition</th>
<th>Factor II</th>
<th>Factor V</th>
<th>Factor VII+X</th>
<th>Fibrinogen</th>
<th>Platelets, 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 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$§</td>
<td>5.1±0.6</td>
<td>420±46§</td>
</tr>
<tr>
<td>Hypercholesterolemic control group, n=8</td>
<td>144±9*</td>
<td>121±10*</td>
<td>142±7*</td>
<td>2.7±0.2$§</td>
<td>550±61§</td>
</tr>
<tr>
<td>Hypercholesterolemic bFGF group, n=8</td>
<td>129±11§§</td>
<td>136±13§§</td>
<td>143±12§§</td>
<td>2.4±0.3§§</td>
<td>576±80§§</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.

### 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.

#### Monocyte TF Activity

In all groups, procoagulant activity was detectable in monocellular cells after 16 hours of culture. A neutralization assay with TF antibody confirmed that procoagulant activity was due to TF expression in all cases. The levels of TF activity in stimulated and unstimulated cells in the 4 groups are shown in Figure 1.

#### 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 (129±45 versus 19±3 μU/1000 monocytes, P<0.05) but was not significant in stimulated cells (124±38 versus 50±11 μU/1000 monocytes, P=NS).

#### 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 μU/1000 monocytes, P<0.05). TF activity in stimulated cells was the same in the 2 groups (53±12 versus 50±11 μU/1000 monocytes, P=NS).

#### Effect of bFGF Administration in Hypercholesterolemic Rabbits

TF activity was higher in the hypercholesterolemic bFGF group than in the hypercholesterolemic control group. This difference was significant in unstimulated cells (31±12 versus 7±1 μU TF/1000 monocytes, P<0.005) and in stimulated cells (185±26 versus 53±12 μU 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 μU TF/1000 monocytes, P<0.05).

#### 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 as 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.

#### 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-
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. 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. 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. 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 mechanism leading to AP-1 and nuclear factor-κB transcription factor activation. Because this pathway is also stimulated by growth factors and serum, 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 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 group and others. 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 lysophosphatidylcholine but is increased by oxidized LDL and free cholesterol. 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 matrix and is associated with cell surface membranes by heparan sulfate proteoglycan (HSPG) binding. Moreover, HSPGs are necessary for the bFGF binding to its receptors to exert both autocrine and paracrine effects. But atherosclerosis involves a decrease of HSPG. 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. 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. TF is also involved in embryogenesis and promotes metastasis of melanoma cells. It was recently demonstrated that the TF–factor VIIa complex can also induce VEGF production. 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. 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 bFGF and TF.

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-
lesterolerolesic rabbit model has been demonstrated to have features in common with humans, and its reproducibility has been demonstrated in several previous studies. This model is useful to understand the processes occurring in humans and to anticipate the effects of bFGF on human blood cells and arteries.

Administration of angiogenic growth factors is an interesting new therapeutic approach for ischemic heart disease. Administration of bFGF improves collateral vessel formation in various models of ischemia. 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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