Soluble Tissue Factor Induces Coagulation on Tumor Endothelial Cells In Vivo if Coadministered With Low-Dose Lipopolysaccharides

Jana Philipp, Ariane Dienst, Maike Unruh, Anke Wagener, Andrea Grunow, Andreas Engert, Jochen W.U. Fries, Claudia Gottstein

Objective—This study was performed to evaluate the mechanisms leading to tumor vessel occlusion by tissue factor–based drugs, which are used in vascular targeting approaches for the treatment of malignant tumors.

Methods and Results—The effects of nontargeted soluble tissue factor were evaluated in vitro and in vivo. Tumor-bearing mice were treated with (1) the extracellular portion of tissue factor (soluble tissue factor), (2) low nontoxic doses of lipopolysaccharides, or (3) a combination thereof. The combination treatment showed the best effects and resulted in selective thrombosis of tumor vessels. On the basis of our data from subsequent in vitro analyses, including surface plasmon resonance measurements and endothelial cell based coagulation assays, we propose a model on how soluble tissue factor, although lacking its membrane anchor, can promote selective tumor vessel occlusion.

Conclusions—To our knowledge, this is the first report to describe the molecular mechanisms of coagulation induction by untargeted soluble tissue factor in vivo. Combination treatments including soluble tissue factor might represent an alternative vascular targeting approach for the treatment of malignant tumors. (Arterioscler Thromb Vasc Biol. 2003;23:905-910.)

Key Words: soluble tissue factor ■ tumor endothelium ■ lipopolysaccharides ■ coagulation ■ vascular targeting

Selective occlusion of tumor vasculature has been proposed as a new approach for cancer therapy. Selectivity is achieved by active targeting of effector molecules to the tumor vasculature and/or by exploiting the procoagulant nature of tumor vessels versus normal vessels (see review1). In an effort to understand the mechanisms of this procoagulant status, we have studied the effects of nontargeted tissue factor (TF) in vitro and in vivo.

TF is the key initiator of the extrinsic coagulation cascade. It is a transmembrane glycoprotein containing 263 residues with a molecular mass of ≈47 kDa and belongs to the cytokine receptor family group 2.2 The extracellular domain of TF is composed of the first 219 amino acids and has been named soluble TF (sTF) or truncated TF. The ability of sTF to induce coagulation compared with full-length TF is greatly reduced.

Lipopolysaccharides (LPS) are constitutive components of the outer membrane of Gram-negative bacteria and are released as endotoxins when the bacteria die or multiply.3 They play a central role in the pathogenesis of Gram-negative sepsis. Many of the effects are explained by their ability to induce immune cells to release cytokines, e.g., tumor necrosis factor (TNF)-α, but direct effects on endothelial cells have also been reported.4 It has long been recognized that LPS can activate the coagulation system. The sensitivity to LPS is very much dependent on the respective species, and compared with other species, mice have low sensitivity to the effects of LPS. Most murine models of LPS-induced shock require doses between 100 and 600 μg and the coadministration of additional factors.5–7

We speculated that LPS would be able to increase the procoagulant activity of tumor endothelium, rendering tumor vasculature more sensitive to thrombosis induction by, for example, sTF. Therefore, we initiated a study to explore in vivo whether LPS would enhance the ability of sTF to induce coagulation in tumor and normal vessels. The possible molecular mechanisms of this thrombosis induction were then further investigated in coagulation assays performed in vitro on the surface of endothelial cells.

Methods
Reagents, Cell Lines, and Mice
LPS from Escherichia coli serotype 055:B5 were from Sigma-Aldrich. L540rec is a human tumor cell line originally derived from a Hodgkin’s lymphoma patient8 and passaged in vivo for increased metastatic potential. F9 murine teratocarcinoma cells were from ECACC. bEnd 3 cells are murine endothelial cells, which can be activated on stimulation with cytokines, and were a kind gift of Dr B. Engelhardt (Max-Planck-Institute, Bad Nauheim, Germany). 2F2B
mouse endothelial cells, expressing constitutively the vascular cellular adhesion molecule-1, were purchased from ATCC/LGC. Tissue culture reagents were from Invitrogen/GIBCO Life Technologies. Molecular biology reagents were from Roche, Fox Chase SCID mice® and BALB/c nude mice were from Taconic/MkB (Ry, Denmark).

**Generation of Recombinant TF Mutant**

Cloning of the gene encoding for the first 219 amino acids of TF and the generation of an expression vector (pswc7) for secretion of sTF into the periplasm of E coli have been described.a sTF was expressed and purified essentially as in Gottstein et al.a To remove endotoxin, we used an affinity resin specific for endotoxins (Dimaco, Isnef). Concentration and purity of the recombinant protein were assessed by SDS-PAGE and scanning UV spectrophotometry. Endotoxin concentrations were measured by a standard LAL assay (Biowhit-taker) according to the manufacturer’s instructions. The coagulation activity of the recombinant protein was confirmed by a cell-free coagulation assay (see method below).

**Coagulation Induction of sTF and LPS In Vivo**

**Animal Models**

The effect of sTF and LPS on tumor vasculature was analyzed in a human xenograft model (L540 cells) and a syngeneic tumor model (F9 cells) in mice. Cells (1×10⁴) were injected subcutaneously into the right flank of SCID or BALB/c nude mice and measured with a caliper in 3 perpendicular directions: a, b, and c. Volumes were calculated according to the following formula:

\[ V = \frac{a \times b \times c}{2} \]

**Treatment Studies**

Treatment was initiated when subcutaneous tumors reached a size of 150 to 300 mL. Reagents were administered into the lateral tail vein. Mice bearing human Hodgkin’s lymphoma were divided into 6 different treatment groups: (1) diluent (0.9% NaCl solution, clinical grade), (2) recombinant depyrogenated sTF at 4 μg total dose, (3) LPS at 0.01 μg total dose, (4) LPS at 0.5 μg total dose, (5) sTF as in group 2, spiked with 0.01 μg LPS, and (6) sTF as in group 2, spiked with 0.5 μg LPS. Mice with murine teratocarcinoma were divided into 4 treatment groups: (1) diluent (0.9% NaCl solution, clinical grade), (2) recombinant depyrogenated sTF at 7 μg total dose, (3) LPS at 0.5 μg total dose, and (4) sTF as in group 2, spiked with 0.5 μg LPS.

Mice were closely observed after treatment for clinical signs of toxicity, and clinical status was documented at defined time points (5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 24 hours, 48 hours, and 72 hours). Three days after treatment, the mice were euthanized, and an autopsy was performed to document any changes in gross pathology. Tumors, lymph node metastases, and the major normal organs (heart, lung, brain, liver, kidney, colon, spleen, and pancreas) were harvested and prepared for histological analysis.

**Histological Evaluation**

Tissue samples were fixed in 3% normal buffered formalin and stained with hematoxyl and eosin (H&E). Light microscopy analysis was performed by 2 independent investigators, with 1 of them being a pathologist. The pathologist was blinded as to which treatment group the animals belonged. Tumor sections with necrotic areas were scanned with a GS-700 imaging densitometer (Bio-Rad), and areas of necrosis were calculated as percentage of total section area. Statistical analysis was performed with the use of SPSS software (SPSS Science Software) and by applying the Mann-Whitney U test for unpaired groups.

**TNF-α Serum Levels in Treated Mice**

Blood from lymphoma-bearing mice treated with 0.5 μg/mL LPS, sTF, or a combination treatment was sampled at 1 hour, 2 hours, and 24 hours after treatment, and serum was prepared. TNF-α levels in serum were determined by using the Quantikine-M kit (R&D Systems) according to the manufacturer’s instructions.

**Cell-Free Coagulation Assay**

A 2-stage coagulation assay based on the ability of TF to activate factor X was performed. Negatively charged phospholipids at a final concentration of 50 μmol/L (phosphatidylcholine and phosphatidylserine [Sigma Chemical Co] at a ratio of 70:30) in calcium buffer (50 mmol/L Tris [pH 8.1], 150 mmol/L NaCl, 2 mg/mL BSA, and 5 mmol/L Ca²⁺) were mixed by extensive vortex mixing with factor VIIa (Haemochrom) at 10 mmol/L and with sTF at different concentrations with or without LPS (10 μg/mL). After incubation for 5 minutes at 37°C, factor X (Sigma) was added to a final concentration of 30 mmol/L, and samples were incubated for 5 minutes at room temperature. Finally, the chromogenic substrate S2765 (Haemochrom) at pH 8.0 was added. Factor Xa generation as a measure of TF activity was determined by the increase in the absorption at 405 nm.

**Binding Analysis by Surface Plasmon Resonance**

Real-time binding analysis, measuring surface plasmon resonance (Biacore), was performed to detect whether sTF in a cell-free environment would be able to bind or adhere to immobilized TF. To this end, sTF was immobilized on a CM5 sensor chip (Biacore) either directly by amine coupling or was captured by a covalently linked anti-human TF antibody. Directly coupled sTF was immobilized at a surface density of 700 response units (RU), the capturing antibody was immobilized at a surface density of 700 RU, and the captured sTF was bound at a density of 300 RU. sTF was then injected at a concentration of 1 μmol/L at a flow speed of 30 μL/min, either alone or after preincubation with LPS (10 μg/mL) or factor VIIa (1 μmol/L).

**Binding of sTF or sTF-VIIa to Endothelial Cells**

Binding on endothelial cells was investigated in a functional assay in which the coagulation activity of endothelial cells preincubated with tissue culture medium (negative control), sTF at 100 nmol/L, factor VIIa at 100 nmol/L, or an equimolar mixture of sTF and factor VIIa (sTF-VIIa) at 100 nmol/L was assessed. bEnd3 cells were seeded in 48-well tissue culture plates at a density of 1×10⁴ cells per well and allowed to adhere overnight. After incubation with medium, sTF, VIIa, or sTF-VIIa for 45 minutes at room temperature, the cells were washed and incubated with a coagulation factor mix containing 50 nmol/L factor IX (Sigma), 60 nmol/L factor X, and 50 μmol/L phospholipids in calcium buffer (as specified above). Neither factor VIIa nor TF was present in the coagulation factor mix. Supernatant of the wells was then transferred into a 96-well ELISA plate. Substrate S2765 was added at pH 8.0. Factor Xa generation as a measure of TF activity was determined by the increase in the absorption at 405 nm.

**Coagulation Induction by sTF-VIIa on Activated Endothelial Cells**

bEnd 3 cells were seeded in 48-well tissue culture plates at a density of 1×10⁴ cells per well and allowed to adhere overnight. Cells were stimulated with LPS (0.5 μg/mL and 10 μg/mL) or tissue culture medium (negative control) for 4 hours at 37°C. Then the cells were washed and subsequently incubated with buffer (negative control), 100 nmol/L sTF, or 100 nmol/L sTF-VIIa equimolar complex for 45 minutes at room temperature. In addition, some samples of sTF or buffer were incubated overnight at 4°C. Cells were then washed and incubated with a coagulation factor mix containing 10 nmol/L factor VIIa, 50 nmol/L factor IX, 60 nmol/L factor X, and 50 μmol/L phospholipids in calcium buffer (as specified above). Supernatant of the wells was transferred into a 96-well ELISA plate. Substrate S2765 was added at pH 8.0. Factor Xa generation as a measure of TF activity was determined by the increase in the absorption at 405 nm. Background values (incubation with buffer) were subtracted to exclude differences produced by endogenous TF expression.

To assess whether or not de novo generation of factor VIIa was promoted, we performed the assay in an analogous fashion; however, we used a limiting amount of factor VIIa in the coagulation factor mix: instead of 10 nmol/L factor VIIa, a mixture of 40 nmol/L factor VII (Calbiochem-Novabiochem) and 0.02 nmol/L factor VIIa was used.
used, allowing the system to generate more factor VIIa from factor VII. The readout was again factor Xa generation. Control reactions that contained only factor VII at 40 nmol/L or only factor VIIa at 0.02 nmol/L were performed. From the amount of factor Xa generated in the reactions with the mix of 40 nmol/L factor VII plus 0.02 nmol/L factor VIIa, background activity (buffer controls) was subtracted as well as the amounts of factor Xa generation produced by the samples incubated with the control mixes (factor VII only and 0.02 nmol/L factor VIIa only). The difference was considered to be due to de novo generation of factor VIIa and is shown for the sTF-VIIa-incubated group versus the sTF-incubated group.

**Coagulation Induction by stF-VIIa In Vivo**

An equimolar mixture of sTF (4 μg) and factor VIIa (6.7 μg) was injected intravenously into 4 L540 tumor-bearing mice, when tumors had reached a size of 150 to 300 μL. Five additional mice were injected with the same amount of sTF and factor VIIa plus LPS (0.01 μg or 0.5 μg). Control mice were treated with sTF alone (n=11) or factor VIIa alone (n=2). After 3 days, tumors and organs were harvested, and histological sections were analyzed as described above.

**Results**

**Recombinant Depyrogenated sTF**

Recombinant sTF (amino acids 1 to 219) was extracted from the periplasmic space of transformed E coli and purified close to homogeneity. The recombinant protein had a molecular mass of ≈30 kDa as determined by SDS-PAGE. After the last endotoxin-removal step, no endotoxin was detected in a 1:10 dilution of the final product. The detection limit of the LAL assay was determined to be ≈1 pg/mL (1 IU corresponds to 30 to 100 pg). Functional activity was verified in a cell free 2-stage coagulation assay. The coagulation activities before and after endotoxin removal were identical.

**Coagulation Induction of sTF and LPS In Vivo**

**Clinical Signs and Macroscopic Findings**

Tumor-bearing mice given diluent, endotoxin-free sTF, 10 ng LPS, or sTF with 10 ng LPS showed no clinical signs of toxicity. Mice with 0.5 μg LPS or sTF plus 0.5 μg LPS had mild toxicity symptoms: slight hypocoagulation beginning 15 minutes after intravenous injection and mild general signs such as ruffled fur and hunched posture. Clinical signs of toxicity were reversible. Some L540 tumors treated with LPS and sTF darkened and eventually turned black 1 day after injection. At the time of autopsy, no gross abnormalities were detected in any of the normal organs.

**Histological Findings in Mouse Tumors and Organs**

Tumor tissues of mice treated with the combination of LPS and sTF showed thrombotic vessels and necrotic tumor tissue (Figure 1F). Tissue necrosis was quantified after densitometry of several representative tissue sections (Figure 1A through 1C). Percentages of tumor tissue necrosis for mice in different treatment groups are listed in the Table.

In normal organs of lymphoma-bearing mice, there were virtually no necrotic areas in any of the treatment groups (Figure 2). Out of 59 mice evaluated for toxicity, we saw 3 cases of a very mild inflammatory reaction in the liver (n=2) or lung (n=1) in mice treated with an LPS-containing regimen (n=41). In these mice treated with an LPS-containing regimen, we saw in 9 mice a single microfocal thrombus in the liver (n=5), lung (n=3), or pancreas (n=1), in 1 mouse a large but nonoccluding lung thrombus, and in 1 mouse a small necrotic area in the liver. No dose dependency was observed for LPS-induced toxicities. No histological abnormalities were seen in mice treated with LPS-free sTF or diluent.

**TNF-α Serum Levels in Treated Mice**

TNF-α serum levels were increased in all but 1 mouse treated with a regimen containing 0.5 μg/mL LPS (n=14). One hour after injection, TNF-α levels rose to an average of 2.8 ng/mL (range 0.5 to 7.6 ng/mL). After 2 hours, average TNF-α levels were 0.3 ng/mL (range 0 to 0.8 ng/mL), and after 24 hours, no TNF-α was detectable. In mice treated with sTF containing no LPS (n=6), TNF-α could not be detected in the serum at any of the time points investigated.

**Percentage of Tumor Tissue Necrosis After Intravenous Treatment With sTF, LPS, or Combination Therapies**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>% Necrosis (mean)</th>
<th>SD</th>
<th>P vs Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, L540 tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluent controls</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>...</td>
</tr>
<tr>
<td>sTF (4 μg)</td>
<td>11</td>
<td>1</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>0.01 μg LPS</td>
<td>6</td>
<td>7</td>
<td>12</td>
<td>0.1</td>
</tr>
<tr>
<td>sTF+0.01 μg LPS</td>
<td>5</td>
<td>33</td>
<td>35</td>
<td>0.005</td>
</tr>
<tr>
<td>0.5 μg LPS</td>
<td>13</td>
<td>11</td>
<td>15</td>
<td>0.1</td>
</tr>
<tr>
<td>sTF+0.5 μg LPS</td>
<td>9</td>
<td>33</td>
<td>26</td>
<td>0.002</td>
</tr>
<tr>
<td>B, F9 tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluent controls</td>
<td>5</td>
<td>32</td>
<td>16</td>
<td>...</td>
</tr>
<tr>
<td>sTF (7 μg)</td>
<td>5</td>
<td>41</td>
<td>36</td>
<td>0.8</td>
</tr>
<tr>
<td>0.5 μg LPS</td>
<td>5</td>
<td>53</td>
<td>21</td>
<td>0.1</td>
</tr>
<tr>
<td>sTF+0.5 μg LPS</td>
<td>5</td>
<td>63</td>
<td>26</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Influence of LPS on Coagulation Cascade in a Cell-Free Coagulation Assay
Addition of LPS to sTF in a cell-free coagulation assay did not result in a statistically significant increase of coagulation activity. Therefore, LPS does not enhance the coagulation activity in the absence of cells.

Analysis of sTF Binding to TF by Surface Plasmon Resonance
sTF alone or preincubated with either LPS or factor VIIa did not bind to or homodimerize with immobilized sTF, as measured by surface plasmon resonance (please see online Figure 1, available at www.ahajournals.org).

Binding of sTF-VIIa to Endothelial Cells
Figure 3A demonstrates that both sTF and VIIa adhered to endothelial cells and supported coagulation. In this assay, neither sTF nor factor VIIa was present in the coagulation factor mix applied after incubation with sTF, factor VIIa, or the sTF-VIIa combination followed by a wash step. Therefore, the observed factor Xa generation is due to the binding of both components, sTF and factor VIIa (when applied concomitantly), to the endothelial cells. Preincubation of the endothelial cells with LPS at 0.5 μg/mL increased the ability of the sTF-VIIa mixture to adhere to endothelial cells and activate factor X 1.5-fold (SD 0.45, data from 6 independent experiments, \( P = 0.001 \) by Mann-Whitney \( U \) test for unpaired groups).

Coagulation Induction by sTF-VIIa on Activated Endothelial Cells
Figure 3B illustrates that the sTF-VIIa complex, compared with sTF as a control, supported coagulation in both unstimu-
labeled and stimulated endothelial cells. More factor Xa was generated in LPS-stimulated cells.

A variation of the assay tested for the de novo generation of factor VIIa. Figure 3C shows that the sTF-VIIa complex is able to support de novo generation of factor VIIa in LPS-stimulated endothelial cells. sTF alone was not able to bind to endothelial cells and increase the coagulation activity after stimulation with LPS. Even after overnight incubation with sTF, there was no increase of coagulation activity in LPS-treated endothelial cells versus control cells.

Coagulation Induction by sTF-VIIa In Vivo
To determine whether coagulation induction by the sTF-VIIa complex would also take place in vivo, the sTF-VIIa mixture was injected intravenously into 4 LS40 tumor-bearing SCID mice. Three of 4 mice showed thrombotic blood vessels in the tumor accompanied by tumor tissue necrosis (37% on average, SD 42%). The addition of LPS did not increase the amount of tumor necrosis. Virtually no necrosis was seen with either of the 2 components given alone (1% for sTF, SD 3; 0% for VIIa, SD 0%). However, treatment with sTF-VIIa resulted also in thromboses in lung, liver, or heart in all but 1 mouse, resulting in a transmural myocardial infarction in 1 mouse. This supports the notion that in the mice treated with LPS plus sTF, in which side effects were much less pronounced, factor VIIa production occurred locally, at the site of the tumor vessels.

Discussion
We can summarize that intravenous injection of sTF together with small amounts of LPS causes thrombosis induction in tumor vessels with subsequent tumor tissue necrosis. sTF or LPS given as a single agent resulted in a much smaller amount of tumor tissue necrosis. Virtually no tissue necrosis was seen in normal organs.

Tumor necrosis induced by injection of LPS or bacterial extracts has been described by a number of investigators but at much higher doses than have been used in the present study. TNF-α seems to play a role, inasmuch as selective coagulation of tumor vessels has been reported after the application of TNF-α and inasmuch as we found elevated TNF-α serum levels in treated mice. TNF-α is known to be upregulated in macrophages on stimulation with LPS. Both TNF-α and LPS have been reported to upregulate TF in endothelial cells and macrophages. A synergistic effect of TF expression has been described for vascular endothelial growth factor (VEGF) and TNF-α by several independent investigators. Because tumor cells are a major source of VEGF, part of the coagulation selectivity for tumor vasculature seems to arise from this TNF-α–VEGF synergism on TF expression.

Although selectivity of coagulation processes to the tumor vasculature caused by LPS can easily be explained by these and other factors (such as venous stasis and inflammatory cells in the tumor), the role of sTF in coagulation promotion is less obvious. sTF, lacking its transmembrane anchor, cannot, by itself, sufficiently bind to membrane surfaces of endothelial cells nor to upregulated endogenous TF. However, factor VIIa, which is present in minute amounts in the blood, is able not only to adhere to endothelial cells but also to promote the adherence of sTF to endothelial cells, resulting in a functional sTF-VIIa complex on the surface of the membrane. As a consequence, factor X becomes activated to Xa, as well as factor VII to VIIa. Therefore, we conclude that factor VIIa, to some extent, able to replace the anchoring function of the transmembrane domain of TF in vivo. In this setting, the concentration of factor VIIa seems to be a limiting factor. Donate et al have shown that dimerization of sTF molecules enables them to activate factor VII to VIIa, although single molecules of sTF are not able to promote...
factor VIIa autoactivation in a cell-free environment. Donat et al also postulated that the proximity of a TF-VIIa complex to a substrate TF-VII complex is a major determinant for this form of autoactivation, on the basis of earlier studies by Neuenschwander et al. In a setting in which endogenous TF is upregulated (i.e., the surface density of TF molecules is already high), additional binding of stF would lead to a further enhancement of the surface density. This might lead to dimer-like molecules of stF and endogenous TF with the ability to activate factor VII to VIIa. Alternatively, de novo generation of factor VIIa could happen through direct activation by factor Xa on the surface of the membrane. In either case, factor VIIa generation appears to occur locally in the tumor vasculature after treatment with stF plus LPS. This hypothesis is supported by the fact, that on intravenous injection of an stF-VIIa mixture, we also observed the induction of thrombosis in tumor vessels, but (in contrast to the stF plus LPS treatment) we now saw a significant number of thromboses in normal vessels. In addition, TNF-α and other cytokines have been reported to enhance externalization of phosphatidylinerine on the surface of endothelial cells. Phosphatidylinerine is a negatively charged phospholipid that serves as an important cofactor in the coagulation cascade and could promote the improved binding of stF-VIIa to the endothelial surface that we have observed.

On the basis of the data presented in the present study, we suggest a model describing the molecular mechanisms of coagulation induction by stF in vivo as illustrated in Figure 4 (see legend for explanation of the model).

In conclusion, there are 2 ways of selective coagulation induction on the surface of intact endothelial cells: (1) via specific antibody directed targeting and (2) via coagulation induction by stF without a functional targeting moiety when additional factors are present that induce local upregulation of endogenous TF and local generation of factor VIIa. Both ways might be exploited for therapeutic approaches in the treatment of cancer.

Acknowledgments

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References


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Soluble tissue factor induces coagulation on tumor endothelial cells in vivo if co-administered with low-dose lipopolysaccharides.

Submission Type: Original Contribution
Figure I: Real time binding analysis of sTF binding to immobilized sTF.

Sensorgram of three flow cells on a CM5 sensorchip. Bottom line: Blank flow cell as negative control. Middle Line: Flow cell after immobilization of anti-human-tissue factor antibody. Top line: Flow cell after covalent coupling of sTF on the matrix of the flow cell. Arrows: Injections of sTF (1 µM). At the first injection, sTF is captured by the anti-human-tissue factor antibody (middle line). The second injection shows no increase in response units (RUs), i.e. no binding of sTF to covalently bound sTF (upper line) or captured sTF (middle line) compared to the negative control (bottom line).