Heparin Strongly Induces Soluble Fms-Like Tyrosine Kinase 1 Release In Vivo and In Vitro—Brief Report

Julia Searle, Martin Mockel, Stefanie Gwosc, Saul A. Datwyler, Fatimunnisa Qadri, Gesa I. Albert, Fabian Holert, Annette Isbruch, Lars Klug, Dominik N. Muller, Ralf Dechend, Reinhold Muller, Joern O. Vollert, Anna Slagman, Christian Mueller, Florian Herse

Objective—Soluble fms-like tyrosine kinase 1 (sFlt1) is involved in the pathophysiology of preeclampsia and coronary artery disease. Because sFlt1 has a heparin-binding site, we investigated whether or not heparin releases sFlt1 from the extracellular matrix.

Methods and Results—We measured sFlt1 before and after heparin administration in 135 patients undergoing coronary angiography, percutaneous coronary intervention, or both. sFlt1 was increased directly after heparin administration (from 254 to 13 440 pg/mL) and returned to baseline within 10 hours. Umbilical veins and endothelial cells treated with heparin released sFlt1. Heparinase I and III also increased sFlt1. Mice treated with heparin had elevated sFlt1 serum levels. Their serum inhibited endothelial tube formation.

Conclusion—Heparin releases sFlt1 by displacing the sFlt1 heparin-binding site from heparan sulfate proteoglycans. Heparin could induce an antiangiogenic state. (Arterioscler Thromb Vasc Biol. 2011;31:2972-2974.)

Key Words: angiogenesis • angiography • coronary artery disease • heparin • sFlt1

Soluble fms-like tyrosine kinase 1 (sFlt1) is a circulating splice variant of cell-bound vascular endothelial growth factor receptor-1. sFlt1 is increased in preeclampsia.1 sFlt1 can induce endothelial dysfunction and thereby may contribute to cardiovascular disease.2,3 sFlt1 is produced by endothelial cells.4 In mice, sFlt1 levels significantly increased within 30 minutes after coronary occlusion.3 Coronary endothelial cells increased sFlt1 expression within 60 minutes of hypoxia.2 The fourth, Ig-like, loop of the extracellular sFlt1 domain has a high-affinity heparin binding site, and several

Figure 1. A, Soluble fms-like tyrosine kinase 1 (sFlt1) values before and after coronary angiography (CA)/percutaneous coronary intervention (PCI). The time point showing values directly after CA/PCI is divided into CA only (single-dose heparin) and PCI (second dose). n=135, *P=0.0009. In patients with high sFlt1 values before PCI, blood was drawn after heparin administration. B, Placental growth factor (PLGF) and vascular endothelial growth factor (VEGF) before and after heparin and sFlt1/PLGF and sFlt1/VEGF ratios. n=20, †P<0.0001 and ‡P<0.05 vs at admission.

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clinical studies have shown that sFlt1 increases after heparin treatment.  

Materials and Methods
We enrolled 135 consecutive patients scheduled for elective coronary angiography (CA) and percutaneous coronary intervention (PCI) (Deutsches Register für Klinische Studien, DRKS00000278). Patients received intravenous unfractionated (5000–10 000 U) heparin during the procedure. We collected serial blood samples and measured sFlt1 and placental growth factor using 2-step chemiluminescent microparticle sandwich immunoassays (Abbott Diagnostics). Human umbilical cords were incubated with either 5 mL of heparin (1 IU/mL) for 10 minutes, 5 mL of heparinase I and III (each 0.4 U/mL) for 30 minutes, or 5 mL of PBS. Our isolation and culture techniques have been described.  

Figure 2. A, Soluble fms-like tyrosine kinase 1 (sFlt1) release in umbilical veins after heparin and heparinase I+III. *P<0.05 and ‡P<0.001. B, Heparin-treated umbilical veins were stained for sFlt1 (Cy3, orange). Arrows indicate endothelium and lamina elastica (green). C, Mice after heparin treatment. n=6, *P<0.01 vs untreated. D, Serum of treated mice inhibited tube formation compared with control serum. E, Tube quantification. n=6, *P<0.001.
Results
In coronary artery disease patients, the median sFlt1 level at admission was 254 pg/mL. sFlt levels increased more than 50-fold to a median value of 13,440 pg/mL directly after CA/PCI (Figure 1A). Within 6 to 10 hours, sFltI values returned to baseline and remained stable after 12 to 24 hours. sFltI levels were higher in patients receiving a second heparin bolus during PCI than in patients with CA only. Figure 1B shows an elevated placental growth factor level after heparin; the sFltI/placental growth factor ratio shows a shift to an antiangiogenic state. Figure 1B also shows a reduced vascular endothelial growth factor level directly after CA/PCI, corresponding to the high sFltI/vascular endothelial growth factor ratio. Elevated sFltI levels were not related to microparticles or cell debris, as ultracentrifugation did not affect the levels (Supplemental Figure I). sFltI isosforms are shown by Western blot. Soluble endoglin, soluble vascular endothelial growth factor receptor-2, and endothelial protein C receptor were not elevated by heparin. Platelet activation did not occur (Supplemental Figure III). In umbilical veins, heparin and heparinase I + III both induced sFltI release (Figure 2A). Immunofluorescence confirmed sFltI release from endothelium (Figure 2B). Low-molecular-weight heparin led to similar effects (Supplemental Figure IV). In endothelial cells, heparin doubled sFltI concentrations in the medium (Supplemental Figure V). Figure 2C shows elevated sFltI in mouse plasma after intravenous or subcutaneous heparin treatment at different time points. Serum from these mice reduced endothelial cell tube formation in culture (Figure 2D, quantified in Figure 2E).

Discussion
Our study supports the hypothesis that heparin induces an antiangiogenic state by releasing sFltI. In coronary artery disease patients receiving heparin during CA and PCI, sFltI values increased 50-fold shortly after heparin administration. Heparin induces an antiangiogenic state by releasing sFltI, which is bound to heparan sulfate proteoglycans. sFltI is released on heparin treatment because of competitive binding at its heparin binding site. This finding could be relevant in diseases with impaired angiogenesis, such as preeclampsia, cancer, and coronary artery disease. Recently, Sela et al reported that local retention and systemic release of sFltI are both mediated by heparin binding and regulated by heparanase.9 The combined data suggest that a widely used therapeutic agent might influence angiogenesis.

Sela et al induced sFltI secretion from vascular smooth muscle cells and human placental villous explants with heparin.9 They also found increased sFltI levels in 5 pregnant women receiving low-molecular-weight heparin and detected heparanase in the syncytiotrophoblasts and placental syncytial knots, which produce sFltI. In addition to showing marked sFltI increases in a large cohort, we showed that serum from heparin-treated animals affects endothelial cells. The combined data underscore the conclusion a commonly used therapeutic agent might influence angiogenesis.

Several studies have found that heparin induces an antiangiogenic state in vitro. In human vascular smooth muscle cells, heparin decreases proliferation and migration, and increases apoptosis. These effects are mediated by heparin binding and heparanase. In addition, heparin inhibits angiogenesis in vivo, which is consistent with our findings.

Clinical consequences are speculative. However, heparin not only might affect sFltI measurement in patients under heparin treatment but also could have pathophysiological effects. Many patients in whom angiogenesis and sFltI levels are relevant receive longer-term heparin treatment, such as cancer patients with venous thromboembolism, pregnant women with premature labor, a wide range of patients with cardiac diseases, and patients undergoing hemodialysis. Further research will be necessary to explore clinical ramifications.

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Abbott Laboratories funded the patient study. The Deutsche Forschungsgemeinschaft (HE 6249/1-1) funded Dr Herse.

Disclosures
Dr Datwyler is a scientific employee of Abbott Laboratories.

References
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Heparin strongly induces soluble fms-like tyrosine kinase 1 (sFlt1) release in vivo and in vitro

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Supplemental Methods

Patients

We enrolled 135 consecutive patients scheduled for elective coronary angiography (CA) and possible percutaneous coronary intervention (PCI) between July 2007 and October 2008 at the Department of Cardiology, Campus Virchow Klinikum of the Charité Berlin, Germany in the Biomarkers in Cardiology-3 (BIC-3) study. Patients for CA received an intravenous bolus of between 5000 and 10,000 IU of Heparin at the beginning of the CA. In patients requiring PCI, a second bolus of Heparin was administered up to the target dose of 100IU/kg body weight or in specific cases depending on the activated clotting time (ACT), with a target value of > 300s. PCI was performed in 106 of the CA patients with clinically relevant coronary stenoses, 29 patients had a diagnostic CA only. We collected serial blood samples from all patients at 5 time points: at hospital admission (initial plasma), directly before the start of CA, directly after the coronary procedure (10-30 minutes), 6-10 hours and finally 12-20 hours after the procedure (CA/PCI).

Inclusion criteria were hospital admission for elective coronary angiography, a negative troponin value at admission and an age above 18 years. Exclusion criteria were severe anaemia with a haemoglobin value below 10g/dl, a life expectancy < 6 months, a state of shock or other acute illness, a detention by law, withdrawal of patient’s consent and acute vascular occlusion during PCI.

The Ethics Committee of the Charité Berlin (EA2/052/07) approved all aspects of the trial. All patients gave their written informed consent prior to enrolment. The trial is registered in the German clinical trials register (Deutsches Register für klinische Studien, DRKS: DRKS00000278).

sFlt1 and PLGF in human samples

Samples were collected in EDTA plasma tubes, they were immediately placed on ice, processed within two hours and stored at -80°C.
sFlt1 and PLGF were measured in a prototype ARCHITECT (Abbott Diagnostics) assay after completion of the trial from the frozen EDTA plasma samples. Upon thawing, samples were centrifuged for clarification prior to testing.

Both ARCHITECT assays are two-step sandwich immunoassays using chemiluminescent microparticle immunoassay (CMIA) technology. The sFlt-1 immunoassay utilizes two monoclonal antibodies and measures sFlt1 in both the free form and when bound to PLGF or VEGF. The assay range is 15 to 50,000 pg/mL and the intra- and interassay coefficients of variation (CV) ranged from 1.3% to 5.2% and 1.9% to 5.9%, respectively. The assay showed <10% interference in a control sample spiked with 250 U/mL heparin (Sigma).

To test plasma for microparticles and shredded membranes, we performed ultracentrifugation (140,000 g, 1 hour, 4°C).

The PlGF immunoassay utilizes two monoclonal antibodies and measures the free, not bound form of PlGF-1 (with approximately 20% cross-reactivity with the PlGF-2 isoform). The assay range was 1 to 1,500 pg/mL. The intra- and interassay CV ranged from 1.4% to 6.7% and 1.8% to 6.7%, respectively, with a lower limit of detection of 1 pg/mL. The chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of PLGF and sFlt-1 in the sample and the RLUs detected by the ARCHITECT i System optics. The concentration of PLGF and sFlt-1 is read relative to a standard curve established with calibrators of known PLGF and sFlt-1 concentrations.

sFlt1 in HUVEC

Human umbilical cords were cut into pieces of 50 mm length and veins were washed with PBS three times. Veins were incubated with either 5 ml heparin (1 IU/ml) for 10 minutes, 5 ml Heparinase I and III (each 0.4 U/ml; Sigma-Aldrich, Germany) for 30 minutes, 5 ml Low molecular heparin (LMWH) (enoxaparin) or 5 ml PBS as vehicle control for 30 min as indicated.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins by colleganase digest as described previously.1 Cells were cultured and incubated with heparin (1 IU/ml) or vehicle control for 30 minutes as indicated.
**Animals**

Male C57BL/6 mice (Charles River) were treated by intravenous (iv) or subcutaneous (sc) injection with 20 IU heparin or vehicle and were sacrificed after an indicated period. For long period treatment (3 days), mice were injected with heparin sc twice daily (10 IU each). Serum and plasma were collected. Local authorities approved the animal protocols (LaGeSo; 231/10) that complied with criteria outlined by the American Physiological Society.

**Tube formation assay**

Growth factor–reduced Matrigel (BD Bioscience) was placed in the wells of µ-slides (Ibidi, Germany) (10µl per well) and incubated at 37°C for 30 minutes to allow polymerization. HUVEC’s in serum-free media were seeded onto the Matrigel-coated wells (10,000 cells per well) and were treated with 5% serum of either untreated or heparin treated mice. Serum was previously checked for sFlt1 concentrations. After 8 hours incubation (37°C), tube formation was assessed through an inverted phase-contrast microscope at ×5 (Zeiss, Germany). Quantification was done with the WimTube Software (Wimasis, Germany).

**Immunofluorescence**

Frozen rings of umbilical cord were cryo-sectioned with 6 µm thickness and stained with the primary antibodies Anti-sFlt1 (ReliaTech, Germany) and with the Cy3-labeled secondary Anti-rabbit antibody (Jackson-Immunoresearch Laboratories). Preparations were examined under a Zeiss Axioplan-2 microscope. Samples were examined without knowledge of the treatment state. Figure 2B shows representing pictures of a group of 6 individual experiments.

**ELISA**

Soluble Flt1 concentrations in Cell culture media, umbilical vein media and mouse serum and plasma were measured using ELISA (R&D Systems) according to manufacturer protocols.
Plasma VEGF, Endoglin, EPCR, sVEGFR2, and PF4 from patients were measured by ELISA (R&D Systems) according manufacturer protocol.

**sFlt1 enrichment and Western blot**

Patient plasma samples were enriched with sFlt1 by using heparin-agarose beads (Sigma-Aldrich) according to manufacture protocols as described earlier.² Briefly, plasma volumes were adjusted to the sFlt1 concentration determined previously by ELISA. Corresponding volumes (0.2-1.2 ml) were diluted to 2 ml with PBS and incubated with 30 µl packed volume of heparin-agarose beads at 4°C for 2 hours. After centrifugation the pellet was washed three times and finally boiled with 40 µl Laemmli’s solution. After centrifugation (14,000 g, 3 minutes) the protein was used for SDS-gel electrophoresis, followed by Tank-blotting on membrane. The membrane was stained specifically for human Flt-1 with the antibody V4262 (Sigma-Aldrich, Germany)

**Statistical Analysis**

Values for Patients data are shown as median (25th/75th percentile) and appropriate non-parametric tests were applied. Statistical analysis was performed using PASW® statistics V 18.0. Experimental and animal data are presented as means ± SEM. Normality was assessed by Kolmogorov-Smirnov tests. Group differences were tested by one-way ANOVA with Scheffe post-Hoc test, Dunnett-T3 or Kruskal-Wallis-Test and Mann-Whitney-U-Test as appropriate. A value of p<0.05 was considered statistically significant.
Supplemental Results

**Supplemental table I: Patient characteristics.** Patients were admitted for elective coronary angiography (CA) and possible percutaneous coronary intervention (PCI).

<table>
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<th>Characteristic</th>
<th>Patients in total n=135</th>
<th>Patients with CA and PCI n=106</th>
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<tr>
<td>Men %</td>
<td>75.9</td>
<td>78.3</td>
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<td>NYHA class (%)</td>
<td>I (13.3) II (17) III (18.5) IV (5.2)</td>
<td>I (13.2) II (16) III (19.8) IV (3.8)</td>
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<td>CCS class (%)</td>
<td>I (10.4) II (27.4) III (17) IV (17.8)</td>
<td>I (6.6) II (27.4) III (16) IV (16)</td>
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<td>Yes (36.8) ex-smoker (44.3)</td>
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**CAD severity**

<table>
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<tr>
<td>CAD-3 %</td>
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<tr>
<td>Left Main Disease %</td>
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Abbreviations: NYHA class=New York Heart association classification of heart failure
CCS class=Canadian Cardiovascular Society (CSS) Functional Classification of Angina, CAD=coronary artery disease, LMD=left main disease
**Supplemental Figure I.** Plasma of heparinized patients (CA/PCI) was ultracentrifuged and assayed for sFlt1. sFlt1 was only detectable in the primary (un-centrifuged) plasma (open bar) and the supernatant (black bar) but not in the pellet.

**Supplemental Figure II.** Plasma from 4 different patients directly after CA/PCI with Heparin treatment were enriched for sFlt1 and isoforms were processed for Western Blot. Plasma from preeclamptic woman served as control. Heparin treatment leads to solely one isoform with ~100 kDa size.
Supplemental Figure III. Endothelial protein C receptor (EPCR)-, endoglin- and soluble vascular endothelial growth receptor 2 (sVEGFR2)- level in patients plasma at admission compared to direct after CA/PCI and heparin treatment. Platelet activation was investigated by C-X-C motif chemokine 4 (CXCL4). (n=20; * p<0.05 vs. at admission)
Supplemental Figure IV. (A) Umbilical veins treated by Low molecular heparin (LMWH) releases more sFlt1 than these treated by vehicle (PBS). (B) Mice treated by Low molecular heparin (LMWH) for 10 minutes either IV or SC, releases more sFlt1 than these treated by vehicle (PBS). (n=6; *p<0.01; **p<0.05 vs. untreated)
Supplemental Figure V. Human umbilical vein endothelial cells (HUVEC) treated by Heparin releases more sFlt1 than these treated by vehicle (PBS). (n=6; *p<0.01)

Supplemental Figure VI. Umbilical veins treated by conditioned medium of primary trophoblasts (3 day culture) did not release more sFlt1 than these treated by medium control. Trophoblast cells have been described to have high heparinase activity.³
Supplemental Figure VII. Heparin control on Tube formation is shown. Tube formation was done with 5% serum of untreated mice and equal amounts of heparin (compared to amount in heparinized mice) are added to evaluate the effect of heparin. No differences were observed between both groups, stating, that effects showing in figure 2D-E are not influenced by heparin themselves.
Supplemental References