Recombinant human, active site-blocked factor VIIa reduces infarct size and no-reflow phenomenon in rabbits

PAOLO GOLINO,1 MASSIMO RAGNI,1 PLINIO CIRILLO,1 ANNALISA SCOGNAMIGLIO,1 AMELIA RAVERA,1 CHIARA BUONO,1 ANGELA GUARINO,1 ORLANDO PIORO,1 CATELLO LAMBIASE,1 FILOMENA BOTTICELLA,1 MIRELLA EZBAN,2 MARIO CONDORELLI,1 AND MASSIMO CHIARIELLO1

1Division of Cardiology, Department of Internal Medicine, Second School of Medicine, University of Naples, 80131 Naples, Italy; and 2Department of Vessel Wall Biology, Novo Nordisk A/S, 2880 Gentofte, Denmark

Oxygen free radicals induce de novo synthesis of tissue factor (TF), the initiator of the extrinsic pathway of coagulation, within the coronary vasculature during postischemic reperfusion. In the present study we wanted to assess whether TF expression might cause myocardial injury during postischemic reperfusion. Anesthetized rabbits underwent 30 min of coronary occlusion followed by 5.5 h of reperfusion. At reperfusion the animals received (1) saline (n = 8), (2) human recombinant, active site-blocked activated factor VII (FVIIai, 1 mg/kg, n = 8), or (3) human recombinant activated FVII (FVIIa, 1 mg/kg, n = 8). FVIIai binds to TF as native FVII, but with the active site blocked it inhibits TF procoagulant activity. The area at risk of infarction (AR), the infarct size (IS), and the no-reflow area (NR) were determined at the end of the experiment. FVIIai resulted in a significant reduction in IS and NR with respect to control animals (28.1 ± 11.3 and 11.1 ± 6.1% of AR vs. 59.8 ± 12.8 and 24.4 ± 2.7% of AR, respectively, P < 0.01), whereas FVIIa resulted in a significant increase in IS and NR to 80.1 ± 13.1 and 61.9 ± 13.8% of AR, respectively (P < 0.01). In conclusion, TF-mediated activation of the extrinsic coagulation pathway makes an important contribution to myocardial injury during postischemic reperfusion.

Am J Physiol Heart Circ Physiol 278: H1507–H1516, 2000.—Oxygen free radicals induce de novo synthesis of tissue factor (TF), the initiator of the extrinsic pathway of coagulation, within the coronary vasculature during postischemic reperfusion. In the present study we wanted to assess whether TF expression might cause myocardial injury during postischemic reperfusion. Anesthetized rabbits underwent 30 min of coronary occlusion followed by 5.5 h of reperfusion. At reperfusion the animals received (1) saline (n = 8), (2) human recombinant, active site-blocked activated factor VII (FVIIai, 1 mg/kg, n = 8), or (3) human recombinant activated FVII (FVIIa, 1 mg/kg, n = 8). FVIIai binds to TF as native FVII, but with the active site blocked it inhibits TF procoagulant activity. The area at risk of infarction (AR), the infarct size (IS), and the no-reflow area (NR) were determined at the end of the experiment. FVIIai resulted in a significant reduction in IS and NR with respect to control animals (28.1 ± 11.3 and 11.1 ± 6.1% of AR vs. 59.8 ± 12.8 and 24.4 ± 2.7% of AR, respectively, P < 0.01), whereas FVIIa resulted in a significant increase in IS and NR to 80.1 ± 13.1 and 61.9 ± 13.8% of AR, respectively (P < 0.01). In conclusion, TF-mediated activation of the extrinsic coagulation pathway makes an important contribution to myocardial injury during postischemic reperfusion.

Oxygen free radicals are highly reactive molecular species that may attack various cell constituents. It has been shown that a burst of oxygen free radical generation follows restoration of flow after a period of ischemia (39, 40), and it has been proposed that these oxidant species might be responsible for a specific form of reperfusion-mediated tissue injury secondary to lipid peroxidation and other irreversible alterations of cell constituents (1, 21, 26). In addition to their effects on TF expression by endothelial cells, preliminary experiments conducted in our laboratory suggest that oxygen free radicals can stimulate endothelial cells within the coronary vasculature to synthesize and express significant amounts of TF (17).

The extrinsic coagulation pathway is initiated when tissue factor (TF), a 47-kDa membrane-bound glycoprotein, is exposed to flowing blood as a consequence of vascular damage (2). TF complexes with factors VII (FVII) and VIIa (FVIIa), permitting enzymatic activation of factors X (FX) and IX (FIX), the substrates for FVIIa, ultimately leading to the generation of thrombin (30). TF is found across the arterial wall, its activity increasing from the subendothelium to the adventitia (28). Significant TF activity has also been localized in human atherosclerotic plaques (38) and, recently, in atherectomy specimens obtained from patients with unstable angina (3). In addition, we recently showed that a monoclonal antibody against TF not only inhibits intravascular thrombus formation in a rabbit model of recurrent arterial thrombosis (28) but also enhances thrombolysis by tissue plasminogen activator (t-PA) and prevents reocclusion after t-PA discontinuation (29). Taken together, these data indicate that TF exposure after arterial damage plays a role in the pathogenesis of acute ischemic coronary syndromes by initiating intravascular thrombus formation.

Normally, endothelial cells, being in contact with circulating blood, do not express significant TF activity. Some studies, however, have suggested that, under certain conditions, endothelial cells may actively promote coagulation by expressing TF-like procoagulant activity (5, 27). In particular, we recently showed that exogenously and endogenously generated oxygen free radicals can stimulate endothelial cells within the coronary vasculature to synthesize and express significant amounts of TF (17).

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tion of the coagulation during posts ischemic reperfusion of the myocardium. This oxygen free radical-mediated expression of TF, with its attendant activation of the extrinsic coagulation pathway, may have important consequences, inasmuch as this phenomenon might impact on the pathophysiology of posts ischemic reperfusion, particularly in patients with acute myocardial infarction undergoing coronary thrombolysis. To determine whether TF-dependent activation of the coagulation cascade plays a role in influencing the fate of posts ischemic myocardium, we have examined the effects of human recombinant, active site-blocked activated FVII (FVIIai) in an experimental model of myocardial ischemia and reperfusion. The rationale for using FVIIai is that it possesses a higher affinity for TF than does native FVIIa (35), but its active site is blocked and it is not capable of converting FIX and FX to their activated forms, thus inhibiting activation of the coagulation cascade and intravascular thrombus formation at an early step (18). This study provides evidence for a role of TF-mediated activation of the coagulation cascade in myocardial injury during posts ischemic reperfusion and identifies a pharmacological intervention of potential therapeutic importance.

METHODS

Preparation of FVIIa and FVIIai

Recombinant human FVIIa was purified from culture media of a transfected baby hamster kidney cell line, as previously described (35). The active site of recombinant FVIIa was blocked by the addition of twofold molar excess of Phe-Phe-Arg chloromethylketone (FFRcmk). The solution was allowed to incubate for 1 h at 4°C. Unreacted FFRcmk was separated from FVIIai by Q-Sepharose Fast Flow ion-exchange chromatography. FVIIai was eluted with 10 mM CaCl2 and the profile of the eluted peak was narrow and symmetrical. The FVIIai solution was adjusted to a protein concentration of ~2 mg/ml in 10 mMol/l glycylglycine, 150 mmol/l NaCl, and 10 mmol/l CaCl2 (pH 7.4), sterile-filtered, and stored at ~80°C. The residual FVIIai activity in the FVIIai solution was <0.1% when measured in an FVIIa-specific amidolytic assay (35).

In Vitro Characterization of FVIIai

The ability of FVIIai to inhibit the procoagulant activity of the TF-FVIIa complex was measured in vitro in a previous study (35). The concentration of FVIIai in solution needed to reduce the FVIIa-dependent FX activation by 50% (IC50) was 0.045 ± 0.012 nM (29). Furthermore, binding studies on cell membrane-associated TF with use of J82 cells, a human bladder carcinoma cell line expressing TF, showed that under these circumstances the IC50 for FVIIai was 1.1 ± 0.2 nM (35). In rabbits the IC50 values for FVIIai were similar to those obtained in experiments employing human TF (M. Ezban, unpublished observations). Finally, in rabbits the apparent plasma half-life of FVIIai was shown to be ~45 min (18).

Experimental Preparation

A total of 58 New Zealand White rabbits of both genders (3.2-3.8 kg) were studied. The experimental preparation has been described in detail elsewhere (15, 16). Briefly, the animals were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly, intubated, and ventilated with a constant-volume respirator (Harvard Apparatus, Cambridge, MA). Polyethylene catheters were placed into the aorta through the left carotid artery and into a jugular vein for monitoring arterial pressure and administering drugs, respectively. A thoracotomy was performed through the fifth left intercostal space, and the pericardium was opened. A polyethylene catheter was placed into the left atrium through the left atrial appendage for later injections of colored microspheres. The large marginal branch of the circumflex coronary artery was temporarily occluded ~0.3 cm from its origin with a surgical suture snare. Coronary artery occlusion was maintained for 30 min, at which time the ligature was released and reperfusion was allowed for 5.5 h (infarct size studies) or 2 h [intracoronary platelet and fibrinogen accumulation studies and intracoronary TF activity studies]. Systemic arterial pressure (Statham P23 DB pressure transducer) was recorded continuously during the experiment (Gould Instruments).

Protocol I.

INFARCT SIZE STUDIES. At the moment of reperfusion, the animals were randomly assigned to one of the following treatment groups: a control group that received a 5-ml bolus of saline into the left atrium (n = 9), a group treated with FVIIai (Novo Nordisk; 1 mg/kg bolus into the left atrium, n = 8), and a group treated with FVIIa (Novo Nordisk; 1 mg/kg bolus into the left atrium, n = 9). This dose regimen of FVIIai and FVIIa was chosen because we previously showed that this dose of FVIIai was capable of inhibiting intravascular thrombus formation for ~6 h, whereas FVIIa, at the same dose employed in the present study, completely reversed the antithrombotic effects of FVIIai (18). After drug administration, reperfusion was maintained for 5.5 h.

ASSESSMENT OF AREA AT RISK, INFARCT SIZE, AND NO-REFLOW AREA. To estimate the distribution of tissue perfusion at the end of the experiment ("no-reflow" area), the animals received an injection of a 6% solution of thioflavin S (1 mg/kg) via the left atrial catheter according to a previously described technique (22). To permit the assessment of the area at risk of infarction, the coronary artery was reoccluded immediately after injection of thioflavin, and a solution of Monostral blue (E. 1. Upjohn; 1 ml/kg) was injected through the left atrial catheter. The heart was then immediately excised, and the left ventricle (LV) was dissected free from all other structures and weighed. The LV was frozen at ~70°C for 30 min and cut into 8–10 slices parallel to the atrioventricular groove. Contours of the normally perfused myocardium as well as the area at risk, according to the distribution of Monostral blue, were traced onto a transparent plastic sheet. Myocardial slices were then observed under ultraviolet light, and the normally reperfused myocardium (fluorescent) was easily differentiated and separated from the nonreperfused myocardium (nonfluorescent) according to the thioflavin distribution. These areas were also traced onto transparent plastic sheets. The slices were next incubated in a 2% solution of triphenyl tetrazolium chloride (TTC; Sigma Chemical) for 10 min at 37°C to visualize the area of necrosis (11). Again, a transparent plastic sheet was used to trace the contours of the normal (TTC-negative) portion. The inter- and intraobserver variability averaged 3.5 and 2.1%, respectively.

The following variables were calculated by planimetry with use of a computerized system by an investigator who was blind with regard to treatment group assignment: 1) the area at risk of infarction, as a percentage of the LV, assessed at the end of the reperfusion period (Monostral blue distribution); 2) infarct size, as a percentage of the area at risk that actually
evolved to necrosis by the TTC staining criterion; and 3) the percentage of the area at risk that did not receive blood flow at the end of the reperfusion period (no-reflow area).

**Regional Myocardial Blood Flow Measurements.** Regional myocardial blood flow (RMBF) was measured in all rabbits in each treatment group. Different-colored plastic microspheres (blue, red, and yellow; Triton Technology, San Diego, CA) were used to measure RMBF 20 min after occlusion and 10 min and 2 h after reperfusion (23). Microspheres were 15 ± 1 µm and suspended in 10% dextran solution with 0.01% Tween 80. To ensure adequate dispersion, the microspheres were sonicated in an ultrasonic bath for 5 min immediately before use. Approximately 500,000 microspheres were injected (0.5–1.0 ml total volume) into the left atrial catheter. One minute before microsphere injection, reference arterial blood flow withdrawal was begun and continued for 1 min after the injection. Tissue samples (100–300 mg) from the center of the ischemic areas and from nonischemic regions were then recovered according to TTC staining. The microspheres were then recovered from tissue by digestion in a 4 M KOH solution at 72°C for 3 h and from reference blood samples by digestion in 16 M KOH at room temperature for 3 h and subsequent microfiltration according to the instructions provided by the manufacturer (23). The dyes were then recovered from the spheres within a known volume of a solvent (dimethylformamide), and their concentrations were determined by spectrophotometry at optimal wavelengths for each dye according to the manufacturer’s instructions. The composite spectrum of each dye solution was resolved into the spectra of the single constituents by a matrix inversion technique (33). Blood flow to each myocardial sample was calculated by the following formula: RMBF = Fr × Am/Aa, where RMBF is myocardial blood flow (in ml/min), Fr is reference flow (in ml/min), Am is absorbance in myocardial sample, and Aa is absorbance in reference blood sample. Myocardial flow was divided by the sample wet weight and expressed as milliliters per minute per gram.

**Coagulation Studies.** To determine the effects of FVIIai and FVIIa administration on systemic coagulation, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured at baseline and 30 min after drug administration. Blood samples (4.5 ml) were collected in 0.5 ml of sodium citrate (3.8%) and centrifuged at 2,000 min at 4°C to separate the plasma. PT and aPTT were measured in duplicate within 2 h from blood collection.

**Evaluation of Intracoronary Platelet and Fibrin(ogen) Accumulation.** After 2 h of reperfusion, the animals were killed, and the hearts were promptly excised and perfused in a Langendorff apparatus with Krebs solution at a constant pressure of 80 mmHg. Perfusion was maintained for 15 min to ensure a complete washout of radioactivity nonspecifically trapped within the coronary vasculature. hearts were then perfused with 100 ml of a warm (37°C) 2% solution of TTC, then the coronary artery was occluded again and the hearts were perfused with 10 ml of a Monostral blue solution. Thereafter, the LVs were sliced into several sections and cut according to the Monastral blue and TTC staining. This technique allowed us to differentiate the nonischemic (Monastral blue-positive) myocardium, the ischemic but viable (Monastral blue-negative, TTC-positive) myocardium, and the necrotic (TTC-negative) myocardium. These tissue sections were weighed and placed in a gamma counter (Beckman Gamma 8000) to determine the amount of 111In and 125I radioactivity in each sample. The raw radioactivity counts were divided by the tissue wet weight to yield counts per gram of tissue; the intracoronary accumulation of platelets and fibrin(ogen) in the ischemic but viable myocardium, as well as in the necrotic myocardium, was normalized by the accumulation in the normal myocardium, yielding the platelet and fibrin(ogen) accumulation ratios.

Protocol III. **INTRACORONARY TF ACTIVITY STUDIES.** To determine whether myocardial ischemia followed by reperfusion is indeed associated with TF upregulation within the coronary circulation, 15 additional rabbits underwent the following protocol. Five animals were subjected to 30 min of ischemia followed by 2 h of reperfusion, as described above. Five animals were also subjected to 30 min of ischemia and received, immediately before reperfusion, a bolus of FVIIai (1 mg/kg), as previously described; reperfusion was also allowed for 2 h. Finally, in five additional rabbits the suture was passed around the coronary artery but was not ligated; these hearts were left in situ for 2.5 h and served as nonischemic controls. At the end of the reperfusion period, the animals were killed, and the hearts were promptly excised and perfused with Krebs solution (in mM: 117 NaCl, 6.0 KCl, 3.0 CaCl2, 1.0 MgSO4, 5.0 EDTA, 16.7 glucose, 24 NaHCO3, pH 7.4) at 37°C in a Langendorff apparatus. Coronary perfusion pressure was maintained constant at 80 mmHg. After an equilibration time of 30 min, TF activity in the coronary circulation was measured as previously described (17). Briefly, all hearts received an infusion of 1 nmol of FVII (Sigma Chemical) followed by 100 nmol of FX (Sigma Chemical). An aliquot of the coronary effluent collected during infusion of FX (1 ml) was incubated...
with a chromogenic substrate specific for FXa (S2337, Kabi Diagnostica; 0.5 mmol/l) for 30 min at 37°C for the amidolytic assay of FXa production, which was dependent on the formation of an enzymatically active TF-FVII complex. The reaction was stopped by addition of 200 µl/ml of sample of 30% solution of acetic acid. The absorbance of the free chromophore (p-nitroaniline) generated was read in a spectrophotometer at 405 nm. Blanks used samples that contained all the reagents except FX. Purified FXa of known concentration (Sigma Chemical) allowed generation of calibration curves linear down to 2.5 nmol/ml.

Statistical Analyses

Values are means ± SD. ANOVA was used for multiple comparisons among groups. Differences for individual groups were tested with Student’s t-test for unpaired observations with Bonferroni’s correction. For comparisons of hemodynamic variables, as well as RMBF among groups, a two-way ANOVA with a design for repeated measures was used.

RESULTS

Fifty-eight rabbits underwent the surgical procedure. In the infarct size studies, two animals died during coronary occlusion for ventricular fibrillation before treatment group allocation and two additional rabbits died during reperfusion (1 in the control group and 1 in the FVIIa-treated group); in the TF activity studies, three animals died during reperfusion (1 in the ischemia-reperfusion group and 2 in the FVIIa-treated group). No animals died in the platelet and fibrin(ogen) accumulation studies. All the animals that died before completion of the experimental protocol were excluded from subsequent statistical analysis. Therefore, eight, six, and five animals in each treatment group were included in the infarct size, platelet and fibrin(ogen) accumulation, and TF activity arms of the study, respectively.

Hemodynamic Measurements

In all treatment groups, coronary occlusion induced a slight decrease in mean arterial pressure; however, no differences were found among the three groups in heart rates and mean arterial pressures during the course of the experimental period (Table 1).

Assessment of Area at Risk, Infarct Size, No-Reflow Area, and RMBF

Coronary occlusion produced an area at risk of infarction assessed by injection of Monastral blue at the end of the experiment that was similar amongst the three treatment groups (31.6 ± 6.3, 28.2 ± 4.1, and 29.2 ± 5.3% of the LV in control and FVIIa- and FVIIa-treated animals, respectively; not significant).

After 30 min of coronary occlusion and 5.5 h of reperfusion, the amount of the area at risk that evolved toward necrosis averaged 59.8 ± 12.8% in the control group. Administration of FVIIa significantly reduced infarct size to 28.1 ± 11.3% of the area at risk (P < 0.01 by ANOVA), whereas FVIIa administration was associated with a significant increase in infarct size to 80.1 ± 13.1% of the area at risk (P < 0.01 vs. controls and FVIIa-treated rabbits; Fig. 1).

Table 1. Hemodynamic variables during coronary artery occlusion-reperfusion

<table>
<thead>
<tr>
<th>Time After Occlusion, h</th>
<th>Control</th>
<th>FVIIa</th>
<th>FVIIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>165 ± 6</td>
<td>159 ± 4</td>
<td>168 ± 6</td>
</tr>
<tr>
<td>0.5</td>
<td>169 ± 5</td>
<td>163 ± 5</td>
<td>163 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>169 ± 6</td>
<td>161 ± 6</td>
<td>161 ± 5</td>
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<tr>
<td>2</td>
<td>163 ± 5</td>
<td>162 ± 5</td>
<td>165 ± 6</td>
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<td>3</td>
<td>165 ± 5</td>
<td>166 ± 6</td>
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<tr>
<td>4</td>
<td>161 ± 6</td>
<td>164 ± 6</td>
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<tr>
<td>5</td>
<td>160 ± 5</td>
<td>162 ± 5</td>
<td>163 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>160 ± 6</td>
<td>164 ± 4</td>
<td>164 ± 5</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>71 ± 4</td>
<td>72 ± 4</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td>65 ± 5</td>
<td>63 ± 4</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>69 ± 4</td>
<td>71 ± 5</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>73 ± 5</td>
<td>73 ± 5</td>
<td>68 ± 5</td>
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<td>74 ± 6</td>
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<td>70 ± 5</td>
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<td>74 ± 5</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>6</td>
<td>69 ± 5</td>
<td>73 ± 5</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SD. FVIIa, human recombinant active site-blocked activated factor VII; FVIIa, human recombinant activated factor VII.

In control rabbits, 24.4 ± 2.7% of the area at risk showed a perfusion defect, as determined by thioflavin S distribution at the end of the experiment (no-reflow phenomenon). The extent of this no-reflow area was significantly reduced by FVIIa and significantly increased by FVIIa to 11.1 ± 6.1 and 61.9 ± 13.8% of the area at risk, respectively (P < 0.01; Fig. 2).

RMBFs to the nonischemic myocardium were similar in the different treatment groups throughout the study. At 20 min of ischemia, RMBF to the ischemic myocardium was similar in all groups of animals, indicating that the severity of ischemia was similar in the different treatment groups. At 10 min of reperfusion, RMBF increased significantly in all groups, however, in the FVIIa-treated group, this increase was significantly
less pronounced than in the other groups. At 2 h of reperfusion, RMBF to the previously ischemic myocardium significantly decreased in all groups compared with the corresponding values obtained at 10 min of reperfusion. However, in the group treated with FVIIai, the decrease in RMBF was less than in the other groups (Table 2).

Previous studies have established that the amount of the myocardial tissue that shows a perfusion defect during posts ischemic reperfusion is related to various parameters; the most important are extent of the area at risk, magnitude of the infarct size, and amount of the residual collateral flow during occlusion (32). Controlling for these variables allows for more precise assessment of the effects of interventions on the extent of the no-reflow phenomenon. In the present study, when the no-reflow area in control rabbits was correlated with these parameters, a close relationship was observed that fits a multiple linear regression equation: NR (percentage of LV) = −14.62 + 0.75(AR) + 0.07(IS) + 3.69(RMBF) \( [r^2 = 0.98, F \text{ test} = 109.3 \pm 0.37, 0.3, \text{and 3.69 (SE)}] \), where NR is no-reflow area, AR and IS are area at risk and infarct size, respectively (both expressed as a percentage of LV), and RMBF is collateral blood flow (in ml·min\(^{-1}·g^{-1}\)). With \( r^2 = 0.98 \), this model accounted for >95% of the variation in the no-reflow area that was observed in control animals in this study.

The equation coefficients that were obtained from the data of control rabbits in the multiple regression equation were then used to calculate the expected no-reflow areas for individual animals within each intervention group. The actually observed no-reflow areas in rabbits receiving FVIIai were significantly smaller than the expected values calculated by applying the equation obtained from the multiple regression analysis. Indeed, in FVIIai-treated rabbits, for any given expected no-reflow area, the actual observed value was smaller, such that all animals in this group distributed below the regression line obtained for control animals. On the contrary, FVIIa-treated rabbits behaved just the opposite; i.e., for any given expected no-reflow area, the actual observed value was significantly larger, inasmuch as all animals distributed above the regression line of control animals (Fig. 3). Taken together, these data indicate that the reduction in the no-reflow areas observed in FVIIai-treated animals is not entirely accounted for by the reduction in infarct size and may suggest that activation of the extrinsic coagulation cascade during posts ischemic reperfusion contributes to the no-reflow phenomenon.

Coagulation Studies

To study possible systemic effects of FVIIai and FVIIa, PTs and aPTTs were measured in blood samples collected before (baseline) and after FVIIai or FVIIa administration. At baseline, PT and aPTT averaged 8.2 ± 0.6 and 25 ± 3 s, respectively. A slight increase in PT to 9.7 ± 1.0 s was observed after FVIIai administration. This increase, however, did not reach statistical significance.

**Table 2. Regional myocardial blood flow during coronary occlusion and reperfusion**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FVIIai</th>
<th>FVIIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal myocardium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min CAO</td>
<td>1.19 ± 0.22</td>
<td>1.03 ± 0.19</td>
<td>1.27 ± 0.24</td>
</tr>
<tr>
<td>10 min Reperfusion</td>
<td>1.22 ± 0.15</td>
<td>1.10 ± 0.17</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>2 h Reperfusion</td>
<td>1.17 ± 0.18</td>
<td>1.07 ± 0.16</td>
<td>1.22 ± 0.19</td>
</tr>
<tr>
<td>Ischemic myocardium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min CAO</td>
<td>0.09 ± 0.05</td>
<td>0.08 ± 0.05</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>10 min Reperfusion</td>
<td>1.53 ± 0.12</td>
<td>1.65 ± 0.18</td>
<td>1.24 ± 0.14*</td>
</tr>
<tr>
<td>2 h Reperfusion</td>
<td>0.89 ± 0.14</td>
<td>1.23 ± 0.15*</td>
<td>0.72 ± 0.13*</td>
</tr>
</tbody>
</table>

Values are means ± SD in ml·min\(^{-1}·g^{-1}\) tissue\(^{-1}\). CAO, coronary artery occlusion. *P < 0.05 vs. other groups.
significance (P = 0.09 by ANOVA and Student’s t-test with Bonferroni’s correction). aPTT did not change significantly after FVIIai administration. FVIIa administration resulted in a significant shortening in PTs and aPTTs only with respect to the values obtained after FVIIai administration (Fig. 4).

Intracoronary Platelet and Fibrinogen Accumulation

To determine whether ischemia and reperfusion are associated with accumulation of thrombi within the coronary circulation, radiolabeled platelets and fibrinogen were injected into animals subjected to 30 min of ischemia followed by 2 h of reperfusion. In control rabbits, platelet and fibrinogen accumulation ratios (defined as the radioactivity in the ischemic myocardium to the radioactivity in the normal myocardium) averaged 3.4 ± 0.6 and 4.2 ± 0.8, respectively, in the ischemic but viable tissue; an even greater accumulation was observed in the necrotic myocardium. Administration of FVIIai markedly reduced platelet and fibrinogen accumulation in the ischemic but viable myocardium to 1.5 ± 0.6 and 1.8 ± 0.6, respectively (P < 0.01), whereas it had no effect on platelet and fibrinogen accumulation in the infarcted tissue (Fig. 5). This lack of effect of FVIIai on platelet and fibrinogen accumulation in the core of the infarcted tissue is probably the consequence of the fact that platelet accumulation in this area may be caused by factors other than TF exposure, such as endothelial disruption and exposure of adhesive proteins, including collagen and von Willebrand factor.

Intracoronary TF Activity

To determine whether ischemia and reperfusion result in upregulation of TF within the coronary circulation of postischemic hearts, TF procoagulant activity was measured in hearts subjected to ischemia and reperfusion in vivo. In control hearts not subjected to regional ischemia, TF activity was undetectable; in contrast, 30 min of ischemia followed by 2 h of reperfusion induced a marked increase in TF activity, as assessed by the amount of FXa generated within the coronary circulation (Table 3). Interestingly, this increase in TF activity was not observed in hearts subjected to ischemia and reperfusion and receiving FVIIai, indicating that this phenomenon was indeed the consequence of TF exposure in the coronary circulation.

<table>
<thead>
<tr>
<th>TF activity</th>
<th>Control</th>
<th>Ischemia-Reperfusion</th>
<th>Ischemia-Reperfusion + FVIIai</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values</td>
<td>ND</td>
<td>9.4 ± 1.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SD of 5 animals in each group, measured as amount of activated factor X (FXa) generated (nmol/ml). TF, tissue factor; ND, not determined. Control hearts were not subjected to ischemia. Experimental hearts were subjected to 30 min of ischemia + 2 h of reperfusion with and without FVIIai.
DISCUSSION

The main findings of the present study are that inhibition of the extrinsic coagulation pathway by FVIIai resulted in a significant reduction in platelet and fibrinogen accumulation within the postischemic myocardium and a concomitant decrease in infarct size and no-reflow area compared with control animals in this model of myocardial ischemia-reperfusion. Conversely, administration of FVIIa had just the opposite results; namely, it increased infarct size and no-reflow area during reperfusion with respect to control animals. These data indicate that TF exposure and/or expression during posts ischemic reperfusion may activate the extrinsic coagulation pathway within the coronary circulation, leading to alterations in coronary blood flow during reperfusion and, thus, influencing the fate of the ischemic myocardium that will ultimately undergo necrosis.

Accumulating evidence indicates that TF-dependent activation of the coagulation cascade is involved in the formation of intravascular thrombi. For instance, in an experimental model of intravascular thrombus formation, we have shown that blocking TF activity by activator protein-1 (28, 29), a monoclonal antibody against TF, or by FVIIai (18) resulted in a complete inhibition of intravascular thrombus formation. Clinical studies have also recently shown that TF antigen (3) and procoagulant activity (37) are significantly increased in atherectomy specimens obtained from patients with unstable angina compared with specimens obtained from patients with stable angina, suggesting that, in these patients, unstable angina may be precipitated by exposure to TF to flowing blood.

In a recent study from our laboratory (17), we demonstrated that endothelial cells, which normally do not express TF (7), exposed to oxygen free radicals showed an increased transcription of TF mRNA that, in turn, was associated with a increased increase in TF procoagulant activity on the cell membrane. This phenomenon also occurred in intact hearts exposed to oxygen free radicals and, most importantly, in situ hearts undergoing brief episodes of regional myocardial ischemia (5 min) and reperfusion; it was accompanied by a significant reduction in coronary blood flow in the posts ischemic myocardium at 2 h of reperfusion that was completely prevented after TF-procoagulant activity was blocked by activator protein-1 (17). These observations prompted us to test the hypothesis that TF exposure and/or de novo synthesis may contribute to determining the fate of posts ischemic myocardium via alterations in tissue perfusion during posts ischemic reperfusion.

The most important determinants that may influence the fate of ischemic myocardium are the amount of collateral flow during ischemia, the size of the area at risk, and the myocardial oxygen demand (32). The two treatments used in the present study, however, did not change significantly these parameters, inasmuch as collateral flow, area at risk, and the rate-pressure product (a major determinant of myocardial oxygen demand) were similar in the three groups of animals. Furthermore, because FVIIai and FVIIa were administered at the time of reperfusion, one can reasonably exclude any effect of these interventions on the ischemic component of cell damage. Thus other mechanisms should be taken into account to explain the results of the present study, namely, a direct effect of FVIIai and FVIIa on coronary blood flow during posts ischemic reperfusion. Indeed, a significant increase in RMBF 2 h after reperfusion was observed in animals receiving FVIIai with respect to controls, whereas FVIIa administration resulted in a significant decrease in coronary flow at the same time point. Furthermore, we have also observed a significant accumulation of radiolabeled platelets and fibrinogen in control animals at the time when coronary blood flow was observed, i.e., at 2 h of reperfusion. This intracoronary accumulation of platelets and fibrinogen (ogen) was markedly reduced by FVIIai.

In additional experiments we have provided direct evidence that in this model of ischemia and reperfusion a marked increase in TF procoagulant activity occurs, as evidenced by the increased generation of FXa within the coronary circulation. Taken together, these findings are consistent with the hypothesis that TF exposure and/or de novo expression within the coronary circulation may activate the extrinsic coagulation pathway, ultimately leading to a reduction in coronary flow during reperfusion. In this respect, the importance of the additional internal control group represented by the animals treated with FVIIa should also be emphasized. In fact, FVIIa is identical to FVIIai, except its active site is not blocked by FFRcmk. Thus, after FVIIa administration, the extrinsic coagulation pathway is not inhibited and may be normally (or even supernormally) activated when TF is exposed to flowing blood. The observation that FVIIai had opposite effects on infarct size and on the no-reflow phenomenon lends further support to the conclusion that FVIIai did not protect posts ischemic myocardium by a nonspecific mechanism but, rather, by inhibiting TF procoagulant activity.

Of even more interest is the observation, in the present study, that FVIIai resulted in a significant reduction in the no-reflow area, whereas FVIIa significantly increased it compared with control animals. On the basis of our data, in fact, it can be reasonably excluded that the effects of FVIIai and FVIIa on the no-reflow areas were due solely to changes in the infarct size area in the respective treatment groups. If this were the case, the no-reflow areas in FVIIai- and FVIIa-treated groups would have been largely predicted by the multiple regression equation. Indeed, the extent of the no-reflow areas was expected to be significantly smaller and larger in rabbits receiving FVIIai and FVIIa, respectively, than in control animals, because it is well known that the size of the infarct is an important determinant of the magnitude of the no-reflow area (22). Yet, the size of the no-reflow areas that was actually observed in the FVIIai-treated animals showed a further, significant reduction with respect to the predicted values and tended to cluster below the
identity line of the regression equation (Fig. 3). Opposite results were observed in the FVIIa-treated animals; i.e., the actually observed no-reflow areas were significantly larger than expected. These findings can be explained if one assumes that TF-mediated activation of the coagulation cascade within the coronary circulation during postischemic reperfusion might represent an important pathophysiological mechanism in the no-reflow phenomenon.

The no-reflow phenomenon, that is, lack of uniform perfusion to the microvasculature of a previously ischemic tissue, was described in the heart for the first time by Krug et al. (24). Subsequently, Kloner et al. (22) demonstrated in a canine model that the extent of the no-reflow phenomenon is proportional to the time of coronary artery occlusion and, thus, to the infarct size. A number of mechanisms have been proposed to explain the occurrence of the no-reflow phenomenon, including capillary compression by parenchymal cell swelling and interstitial edema, obstruction of small arterioles by endothelial cell swelling and intravascular aggregation of blood cellular components, and constriction of arterioles (2). However, there is no clear indication as to whether any or all of these mechanisms contribute to, are coincident with, or are consequential sequela of the death of cardiac myocytes. Interestingly, Ambrosio et al. (2) demonstrated that the no-reflow area was about three times larger after 3.5 h than after 2 min of reperfusion. Furthermore, direct measurements of blood flow showed that myocardium with poor perfusion at 3.5 h received, for the most part, substantial reperfusion initially and then a marked decline in flow over time (2). These findings demonstrate that, in addition to the “immediate” no-reflow phenomenon at the time of reperfusion, a substantial portion of postischemic myocardium undergoes progressive vascular obstruction during reperfusion (“delayed” no-reflow phenomenon). A major question is, therefore, whether vascular obstruction during reperfusion may directly contribute to myocyte damage. The available evidence would suggest that, at least for the immediate no-reflow phenomenon, the die is cast, and the surrounding myocytes are already dead at the moment of reperfusion (22). However, the situation is less dear for the delayed no-reflow phenomenon. Interestingly, in the present study, direct measurements of regional blood flow revealed a significant decrease in flow in the ischemic myocardium at 2 h of reperfusion; this impairment in flow was significantly reduced by FVIIa treatment, whereas FVIIa further decreased flow to the previously ischemic myocardium. At the same time point, i.e., 2 h after reperfusion, we have shown in additional experiments a significant accumulation of platelets and fibrin(ogen), which was also significantly reduced by FVIIa. These findings, although not conclusive, are compatible with the hypothesis that the delayed component of the no-reflow phenomenon might cause, at least in part, tissue necrosis during postischemic reperfusion, initiating a vicious cycle of reperfusion-induced vascular and myocyte injury.

TF-mediated activation of the coagulation represents only one of the possible mechanisms for the no-reflow phenomenon. For instance, granulocytes have been proposed as a major factor in the no-reflow phenomenon. Studies by Engler et al. (8–10) showed leukocytes in individual capillaries from postischemic myocardium, with red blood cells apparently piled up behind them, suggesting mechanical obstruction. Reperfusion greatly enhances accumulation of neutrophils in the postischemic myocardium in a time-dependent fashion (4, 9), presumably secondary to enhanced adhesion of white blood cells to the capillary endothelium. The hypothesis that leukocytes can cause microvascular obstruction is supported by the observation that depletion of leukocytes or administration of agents that interfere with neutrophil function reduces granulocyte and red cell accumulation and results in less no-reflow area (34). The data of the present study, however, are not necessarily in contrast to the above-mentioned studies: the two phenomena can be, in fact, related to each other, such that TF-dependent activation of the coagulation within the postischemic myocardium might result in potentiation of leukocyte infiltration via activation of platelets and platelet-activating factor production (14). On the other hand, activated leukocytes might favor platelet activation (14), as well as activation of the coagulation via exposure of TF on the surface of activated monocytes (31). Thus, although not directly investigated by the present study, part of the beneficial effect of FVIIa might be due to a reduction of leukocyte infiltration in the postischemic myocardium.

Possible Sources of TF

In principle, the TF responsible for the activation of the extrinsic coagulation cascade during postischemic reperfusion might represent different pools of TF. For instance, one pool of TF is represented by the protein already present in the subendothelial tissue and exposed to flowing blood as a consequence of ischemia; a second pool of TF is the one de novo synthesized by endothelial cells as a consequence of oxygen free radical generation (17). It is likely, however, that both sources are simultaneously operative, although probably with a different timing. In fact, in a similar model of myocardial ischemia and reperfusion not associated with myocardial necrosis (5 min of ischemia followed by reperfusion), we have shown that an impairment in coronary blood flow occurs after 2 h of reperfusion; this phenomenon could be prevented by interfering with the binding of TF to circulating FVII (17). On the other hand, in a canine model of myocardial ischemia and reperfusion associated with significant necrosis, Ambrosio et al. (2) showed that a perfusion defect existed early after reperfusion had been established, suggesting that when myocardial necrosis is present, preformed TF in the subendothelial tissue may play the most important role in the early phase of reperfusion; it cannot be excluded, however, that de novo synthesis of TF by endothelial cells might also participate in the “late” no-reflow phenomenon. Finally, the role of circulating monocytes activated within the coronary circulation of
FVIIa, might have affected infarct size per se. FFRcmk, the substance used to block the active site of FVIIai was administered as a single 10-min infusion. Because the time course of TF expression after ischemia and reperfusion is not known, it is possible that TF might reactivate the extrinsic coagulation pathway once FVIIai is cleared from the circulation, leading to a partial or total loss of the initially observed benefits. However, if this phenomenon is operative, it should not affect the importance of the findings of the present study and could be easily overcome by prolonging the infusion of FVIIai. Finally, it is in theory possible that FFRcmk, the substance used to block the active site of TF, might have affected infarct size per se; this hypothesis, however, seems unlikely, because unbound FFRcmk was completely removed from FVIIai by Q-Sepharose Fast Flow ion-exchange chromatography.

Potential Clinical Implications

Over the past decade there has been intense interest in the concept of treating patients with acute myocardial infarction with reperfusion strategies, including coronary thrombolysis and/or primary angioplasty. In particular, intravenous administration of a front-loaded dose of t-PA has been shown to induce successful recanalization of the infarct-related artery in ~80% of the patients (20). This value can be increased up to >90-95% by primary percutaneous transluminal coronary angioplasty (25). However, not all studies have demonstrated an improvement in LV function after recanalization of the infarct-related artery (12). At the moment, even with the most effective reperfusion strategies, a substantial number of patients exhibit a "low-flow" condition in the infarct-related coronary bed (thrombolysis in myocardial infarction grade II or lower). This condition is associated with an almost complete lack of benefits, at least in terms of mortality (12). It is tempting to speculate that these low-flow conditions might be caused, at least in part, by the inability of blood to reenter all the vasculature of the previously ischemic myocardium and that TF-mediated activation of the coagulation might be, at least in part, responsible for this phenomenon. In this respect, administration of FVIIai to patients with acute myocardial infarction undergoing coronary thrombolysis seems particularly attractive, because this substance not only should accelerate thrombolysis and prevent reocclusion of the infarct-related artery (29) but should also reduce infarct size via a reduction of the no-reflow area. Further studies are warranted to elucidate the potential clinical applications of FVIIai in such a clinical setting.

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Address for reprint requests and other correspondence: P. Golino, Div. of Cardiology, University of Naples “Federico II,” via Sergio Pansini 5, 80131, Naples, Italy (E-mail: golino@unina.it).

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