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Evaluating surface bound rTFPI through an in vitro model of vessel wall injury

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Abstract

Introduction—Injury to the surrounding vessel wall is one of the major reasons for failure of implantable medical devices. The surgical procedure itself or the altered flow conditions after implantation can cause damage to the vessel wall. This damage exposes tissue factor (TF), the initiator of the extrinsic pathway of coagulation. One approach to combat thrombosis is to use an anticoagulant on the surface of the device. The primary aim of this study is to develop a simplified physiologically relevant in vitro model of vessel wall injury to study the mechanisms by which immobilized recombinant tissue factor pathway inhibitor (rTFPI) effectively inhibits TF initiated thrombosis.

Materials and methods—A two well chamber slide was used for the study. Fibroblasts were cultured on the upstream portion of the slide. Fibroblast cells stimulated with TNF- α acted as a source of surface TF. The downstream portion of the slide was coated with rTFPI. A mixture of FX, FVIIa and calcium was perfused over the slides to generate FXa. Effluent collected at the outlet was used to analyze the inhibition of this surface generated FXa by the rTFPI present downstream.

Results and conclusions—Different shear rates and rTFPI densities were used to study this effect. In most cases rTFPI inhibited FXa generated upstream as a function of the wall shear rate and rTFPI dosage (surface density). This study shows the effectiveness of the surface bound inhibitor when FXa is generated from an upstream injury site and the bulk of FXa is near the wall.

Keywords

coagulation; TF; rTFPI; anticoagulant; vessel wall injury

Implantation of devices such as stents or grafts can disrupt the vessel wall adjacent to the device [1–4]. This injury to the vessel wall exposes the tissue factor (TF) normally present in the subendothelium [5]. TF forms an enzymatic complex with circulating factor VII/VIIa and activates factor X to Xa, [6] the first reaction of the common coagulation pathway that triggers a number of subsequent biochemical reactions finally resulting in the formation of a thrombus [7]. This can reduce not only the effectiveness of the device but may contribute to device failure. In order to evaluate the effectiveness of an immobilized FXa inhibitor in minimizing the risk of thrombosis from upstream vessel wall injury, an *in vitro* model was

developed and tested. Previous animal studies demonstrated that passively adsorbed rTFPI can successfully reduce thrombogenicity of Dacron [8–10]. In vitro studies under controlled flow conditions showed that fibrin deposition on Dacron graft materials by non-anticoagulated blood was also reduced by immobilized rTFPI [11]. Presumably, the reduced fibrin deposition was due to inhibition of FXa. Subsequent *in vitro* flow studies using purified proteins demonstrated that rTFPI immobilized on the wall of a parallel plate flow chamber inhibited purified FXa added to the perfusate [12].

The aim of this investigation was to develop a simplified physiologically relevant *in vitro* model of vessel wall injury in which FXa is generated near the injury site, rather than entering the flow system at a constant concentration across the entire flow regime. This will result in a higher concentration of FXa near the wall and will affect the inhibition downstream due to different transport characteristics compared to the previous studies where FXa was present in the bulk. This makes the model more realistic and gives a better representation of the situation arising from injury to the vessel wall *in vivo*. The use of cells as a source of TF makes the model more physiological. It represents injury upstream of the implantation site and studies the mechanism by which immobilized rTFPI effectively inhibits locally generated FXa and hence, TF initiated thrombosis. FXa was generated from TF expressed on the surface of mouse fibroblast cells and subsequent inhibition of this FXa occurred by the immobilized TFPI present downstream. Inhibition was studied using an *in vitro* flow chamber at three different wall shear rates ranging from 50 to 400 sec⁻¹ and for two rTFPI surface densities of 78 and 127 ng/cm² to evaluate the effect of shear rate and rTFPI dosage on FXa inhibition.

Materials and methods

Proteins and buffers

Human FX and FVIIa were purchased from Enzyme Research Laboratories (South Bend, In) and diluted to 5 µM and 500 nM respectively in hepes buffered saline with bovine serum albumin (BSA) (HBSA: 0.01 M hepes, 0.14 M NaCl, 0.1% BSA at pH 7.5). Recombinant full-length human tissue factor pathway inhibitor (rTFPI) was a gift from Chiron Corporation (Emeryville, CA). Required rTFPI concentrations were prepared by diluting the stock in phosphate buffered saline (1-X PBS, pH 7.4, Sigma Chemical Co. St. Louis, MO). 3% bovine serum albumin (BSA) (Fisher Biotech, NJ) was used to block all surfaces to prevent non-specific adsorption of FXa. HEPES buffered saline with ethylenediaminetetraacetic acid (HBSA/ EDTA: 0.05 M EDTA, 0.01 M hepes, 0.14 M NaCl, 0.05% BSA, pH 7.45) was used in FXa assays.

The reaction mixture consisted of 100 nM FX, 10 nM FVIIa, 5 mM CaCl₂ in HBSA. The TNF-α used to stimulate the cells was from Abcam (Cambridge, Ma) and reconstituted to 20 µg/ml with Dulbecco's Modified eagle's Medium (DMEM, Gibco).

Quantification of surface bound rTFPI

The amount of rTFPI adsorbed on the two-well glass chamber slides (Cat # 177380, Fisher Scientific, NJ) was determined using ¹²⁵I labeled rTFPI. Recombinant tissue factor pathway inhibitor was labeled with ¹²⁵I using iodobeads (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Labeled rTFPI was subjected to TCA (trichloro-acetic acid) precipitation to ensure that there was little or no free iodine or degraded rTFPI present. Three different concentrations of rTFPI (20, 40 and 60 µg/ml) were used. Slides were cut into small rectangular pieces and incubated with 100 µl of the rTFPI solution for two hours at 37 °C. Pieces were rinsed three times with 1-X PBS and transferred

into the counting tubes for radioactivity measurement (Packard Inc., Cobra II Autogamma, IL).

Factor Xa chromogenic assay

FXa concentration in the chamber effluent was quantified using a chromogenic activity assay utilizing Spectrozyme-FXa (American Diagnostica, NY). Fifty μl of the effluent sample was added to 50 μl of HBSA/EDTA in a microtiter plate. The plate and spectrozyme were incubated separately at 37 °C for 30 minutes. Fifty μl of 1.5 mM spectrozyme was added to each well and the change in absorbance was measured in a kinetic plate reader (SpectramaxPlus™ from Molecular Devices). A linear standard curve using known concentrations of FXa (0–6 nM FXa) developed previously was used to convert the absorbance readings to FXa concentrations.

Flow Studies

One well of a two-well chamber slide was used for FXa generation and the second well served as an anticoagulant coated surface. Fibroblast cells cultured in the first well acted as a source of TF [13]. The seeding density was 6000–12000 cells/cm² to achieve cell confluence within 2–3 days. On the day of the experiment, cells were stimulated with 10 ng/ml TNF- α added to fresh medium for 3–4 hours at 37 °C. The remaining 50% downstream area was coated with rTFPI or BSA as a control for the same amount of time. At the end of the incubation period medium and rTFPI/BSA were removed and the wells were washed with PBS before placing the slide in the parallel plate flow chamber.

The reaction mixture containing FX, FVIIa and calcium was perfused over the slide and the effluent collected at the outlet was assayed for FXa. Control (no rTFPI) and treated (with rTFPI) experiments for a particular shear rate and rTFPI density were done on the same day using the same batch of cells. FXa generated from the control slides appeared in the effluent depending on its residence time in the flow system. Delay in the appearance of FXa in the effluent corresponded to the inhibitory effect of the rTFPI coated surfaces. This delay was used to evaluate the inhibitory activity of rTFPI. The molar flow rate (moles/min) of FXa exiting the chamber was calculated from the FXa molarity multiplied by the volumetric flow rate. The percent of FXa inhibited relative to the control as a function of time was also determined.

Results

Quantification of adsorbed rTFPI

The surface density of adsorbed rTFPI increased linearly with increasing rTFPI solution concentration without apparent saturation. The surface density of adsorbed rTFPI was 78.5 ng/cm², 127.2 ng/cm² and 170 ng/cm² from solutions of 20, 40 and 60 $\mu\text{g/ml}$ respectively (Table 1). The differences were statistically significant for all the concentrations used (One-way-ANOVA, $p < 0.05$).

FXa inhibition by rTFPI

The inhibition of FXa by immobilized rTFPI was studied at two shear rates (50 and 200 sec⁻¹) at a surface density of 78 ng/cm² and at three shear rates (50, 200 and 400 sec⁻¹) and a surface density of 127 ng/cm². In all cases, the presence of rTFPI reduced the molar flow rate of FXa in a wall shear rate and rTFPI density dependent manner. The molar flow rate (fmole/min) of FXa exiting the chamber as a function of sampling time was compared for the control (0 ng/cm² rTFPI) and the treated surface (78 ng/cm² or 127 ng/cm²) at each wall shear rate (Figs. 1–3). Student's *t*-test was used to establish significance ($p < 0.05$). Results are reported as mean $\pm$ standard error of the mean. In the absence of rTFPI there is a transient

phase followed by a steady state regime as FXa traverses the chamber and appears in the effluent. The level of the steady state molar flow of FXa is dependent on the extent of FXa generation by TF present on the cells and the time to achieve this level decreases with increasing wall shear rate. The rTFPI treated surfaces significantly reduced FXa in the effluent at nearly all time points (data pairs not statistically significant are noted with an asterisk) and there does not appear to be a saturation of the coated surfaces as a steady state is not achieved during the time course of any experiment. At the higher rTFPI density (127 ng/cm²) and the lowest wall shear rate of 50 sec⁻¹, the difference between the control and rTFPI treated is most obvious. There is an increased generation of FXa with increased shear rate and for a fixed surface concentration the difference becomes less obvious. As a result, the higher rTFPI density was examined at 400 sec⁻¹ whereas the lower density was tested up to 200 sec⁻¹.

The difference in the moles/min of FXa exiting the chamber in the presence and absence of rTFPI was compared to determine the % inhibition of FXa. Clearly, as the sampling time increases, the rTFPI binds FXa reducing the available sites and more FXa therefore appears in the effluent. However, there appears to be a fairly consistent decrease in comparison to the control over the time period of each case.

Fig. 4 shows the effect of rTFPI surface density on FXa inhibition at wall shear rates of 50 and 200 sec⁻¹. It was found that increasing the surface density by ~60% increased the inhibition of FXa on average 23% for a wall shear rate of 50 sec⁻¹ and 16% on average for a wall shear rate of 200 sec⁻¹. Fig. 5 demonstrates the effect of increasing wall shear rate on FXa inhibition. At 127 ng/cm² rTFPI three wall shear rates were examined. A factor of 2 decrease (400 to 200 sec⁻¹) improved inhibition by 20%. A 4x decrease to 50 sec⁻¹ resulted in 40–45% more inhibition.

Discussion

In addition to presenting a potentially procoagulant surface, implanted stents, grafts and other devices tend to cause injury to the surrounding vessel wall and expose TF that is normally separated from the circulating blood by an endothelial cell barrier [14–17]. Exposure of this subendothelial TF may ultimately lead to the formation of thrombus and can contribute to device failure. The initiation of coagulation at the vessel wall means that local concentration of coagulation products such as FXa may be high whereas the bulk concentration may not be significant. Therefore, the efficacy of surface bound rTFPI in the inhibition of FXa generated due to vessel wall injury as a means of abrogating thrombosis was studied. In this model we have coupled previous studies involving interactions of bulk FXa with immobilized rTFPI with a more realistic transport scenario. Early *in vitro* experiments involved a single step in which FXa entering the system diffused to the TFPI on the surface and was inhibited. However this model involving transport of FX and FVIIa to the surface with TF, formation of the TF:FVIIa complex, conversion of FX to FXa by this complex and subsequent inhibition of this FXa by rTFPI mimics the *in vivo* situation much more accurately than the previous studies. *In vivo* experiments are very useful in terms of determining if a biomaterial coating has any potential but *in vitro* data are required to quantify the *in vivo* results. These quantitative evaluations of the coating *in vitro* and under physiological flow conditions can be used for optimizing the surface prior to implantation in animal models. The performance of the coatings relative to the local flow conditions and coating density was examined. Specifically, the effect of wall shear rate, a determinant of mass transport near the wall was evaluated.

The increase in FXa inhibition was a function of rTFPI surface density, implying a specific reaction, and a function of wall shear rate, implying diffusion of reactants was a strong

determining factor at all conditions studied. At any given wall shear rate, the effect of increasing surface density was similar within the range studied. This implies a specific interaction between immobilized rTFPI and circulating FXa, as previous work also determined (Chandiwal). At (50 and 200 sec⁻¹) when the surface density was 78 ng/cm², the initial inhibition was approximately 60–70% and with time decreased to 15–20%. However when the density was increased to 127 ng/cm², the inhibition was 75–85% initially and at the end of the experiment the FXa inhibition reduced to 30–40% of the inlet concentration. The extent of FXa generated upstream is dependent on the delivery of FX to the TF:VIIa complexes on the cell surface and on the rate of the enzyme catalyzed reaction. The shear rate dependence indicates that the overall process is controlled by the delivery of FX, or it is, in part, “diffusion controlled”. This is also the case for the delivery of FXa to the rTFPI coated surface. As the shear rate is increased the delivery of FXa to the surface also increases resulting in rapid saturation of the rTFPI surface. At both coating densities (78 and 127 ng/cm²), the inhibition was more apparent at the lower shear rates. At a shear rate of 50 sec⁻¹ the percentage inhibition of FXa was maximum. As the shear rate was increased to 200 sec⁻¹ and then to 400 sec⁻¹ for 127 ng/cm² of rTFPI the percentage of FXa inhibition at all time points gradually decreased, as expected.

Most published studies have examined rTFPI as an inhibitor in the fluid phase. Two previous studies from this lab group examined the inhibition of fibrin deposition from whole blood and the inhibition of FXa by passively adsorbed and covalently bound rTFPI. In this second study, purified FXa was perfused at 10 and 20 nM over coated surfaces and the decrease in FXa in the effluent quantified the extent of inhibition. However, the concentration in the bulk was not necessary representative of the FXa concentration profile expected *in vivo* by surface production of FXa. In the current study, FXa was generated in a more physiologic manner using TF expressing fibroblasts present at the vessel wall *in vivo* and perfusing purified FVIIa and FX over them. The FXa was generated at the wall such that local concentrations were more relevant and the effect of a surface bound inhibitor on the same wall could be determined.

In summary rTFPI coating can be an effective method of reducing thrombosis related to vessel wall injury. However its efficacy is dependent on the flow rate and the surface density. Different flow conditions prevail in different parts of the vascular system so depending on the site of implantation the surface density will have to be adjusted to get the optimum result. At present there is no satisfactory treatment for device related thrombosis arising from injury to the vessel wall. Therefore this coating can be a promising approach for solving such problems.

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Fig. 1.

Inhibition of surface generated FXa. FXa in the effluent as a function of time and rTFPI surface density at 50 s^{-1} (A) control for 78 ng/cm^2 rTFPI density (□); in the presence of 78 ng/cm^2 rTFPI density (○) (B) control for 127 ng/cm^2 rTFPI density (■); in the presence of 127 ng/cm^2 rTFPI (●). * Statistically not significant.

Fig. 2. Inhibition of surface generated FXa. FXa in the effluent as a function of time and rTFPI surface density at 200 s^{-1} (A) control for 78 ng/cm^2 rTFPI density (□); in the presence of 78 ng/cm^2 rTFPI density (○) (B) control for 127 ng/cm^2 (■); in the presence of 127 ng/cm^2 rTFPI (●). * Statistically not significant.

Fig. 3. Inhibition of surface generated FXa. FXa in the effluent as a function of time at 400 s^{-1} and rTFPI surface density of 127 ng/cm^2 : control (■); in the presence of rTFPI (●). * Statistically not significant.

Fig. 4. Effect of surface density on FXa inhibition (A) 50 s⁻¹ and 127 ng/cm² rTFPI (■); 78 ng/cm² rTFPI (●) (B) 200 s⁻¹ and 127 ng/cm² rTFPI (□); 78 ng/cm² rTFPI (○).

Fig. 5. Effect of shear rate on FXa Inhibition. Data represents adsorbed rTFPI at a surface density of 127 ng/cm²: 50 s⁻¹ (○); 200 s⁻¹ (●); 400 s⁻¹ (▲).

Table 1

Recombinant TFPI adsorption on chamber slides. The table shows amount of rTFPI adsorbed on the slide as the rTFPI solution concentration is increased.

rTFPI Concentration ($\mu\text{g/ml}$)	Adsorbed rTFPI (ng/cm^2)
20	78.48
40	127.14
80	169.73