Efecto de la inhibición de la vía del factor tisular en la respuesta plaquetar a la lesión vascular

Effect of tissue factor pathway inhibition on platelet response to vascular damage

Sánchez S., Vilahur G., Casani L., Badimon L.

RESUMEN

Antecedentes: El FFR-rFVIIa es un nuevo factor VIIa recombinante con su centro activo inhibido, que compite con el factor VIIa (FVIIa) por la unión al factor tisular (TF). El TF está relacionado con el gran potencial trombogénico de las placas ateroscleróticas.

Objetivo: Evaluar el efecto inhibitorio del FFR-rFVIIa sobre la formación del trombo desencandenado por pared vascular lesionada en condiciones hemodinámicas controladas.

Métodos y resultados: Hemos estudiado la trombosis desencadenada por pared vascular lesionada ligera y severamente y su inhibición en un modelo experimental *ex vivo* porcino utilizando la cámara de perfusión Badimon a una velocidad de cizalladura de 212 seg^{-1}. Los animales ($n = 12$) fueron anestesiados, heparinizados y cateterizados. A continuación se montó un circuito extracorpóreo (arteria carótida-vena yugular) en el que se situó la cámara de pefusión. Después de las perfusiones basales, los cerdos se trataron con FFR-rFVIIa (0,5 y 1 mg/kg i.v.) y se obtuvo una reducción de la deposición plaquetaria sobre lesión vascular severa ($p < 0,005$), sin observarse efecto sobre lesión vascular ligera. Las determinaciones plasmáticas mostraron que el tratamiento con FFR-rFVIIa (0,1, 0,5, 1 y 2 mg/kg) alargaba el tiempo de protrombina (PT) ($p = 0,0001$), y que este parámetro se correlacionaba positivamente con los niveles de FFR-rFVIIa ($r^2 = 0,82$; $p = 0,0001$).

Conclusiones: La inhibición de la vía del TF mediante FFR-rFVIIa reduce significativamente la deposición plaquetaria sobre lesión vascular severa. Este efecto antitrombótico se acompaña de un alargamiento dosis-dependiente del PT. Tales resultados sugieren que la inhibición del TF puede ser una estrategia prometedora en el tratamiento de la aterotrombosis.

Palabras clave: *FFR-rFVIIa, factor tisular, factor VIIa, plaquetas, trombosis, fibrina, deposición plaquetaria.*

Efecto de la inhibición de la vía del factor tisular en la respuesta plaquetar a la lesión vascular
Sánchez S, Vilahur G, Casani L, Badimon L
Investigación Cardiovascular 2003; 6: 42-53

ABSTRACT

Background: FFR-rFVIIa is a new recombinant active site inhibited factor VIIa molecule that competes with factor VIIa (FVIIa) for tissue factor (TF) binding. TF accounts for most of the thrombogenic potential of atherosclerotic plaques.

Objective: To evaluate the inhibitory effect of FFR-rFVIIa on thrombus formation triggered by damaged vessel wall under controlled flow conditions.

Methods and results: Thrombosis triggered by eroded and severely damaged vessels and its inhibition were studied in an *ex-vivo* experimental porcine model, using the Badimon perfusion chamber at a local blood shear rate of 212 sec^{-1}. Animals ($n = 12$) were anesthetized, heparinized, catheterized, and the perfusion chamber was placed in an extracorporeal shunt (carotid artery-jugular vein). After baseline perfusions, pigs were administered FFR-rFVIIa (0.5 and 1 mg/kg i.v.). Platelet deposition on severely damaged vessels was significantly inhibited ($p < 0.005$) by FFR-rFVIIa, but no effect was found on eroded vessels. Plasma determinations showed that prothrombin time (PT) was significantly prolonged with FFR-rFVIIa treatment (0.1, 0.5, 1 and 2 mg/kg) ($p = 0.0001$), and changes in PT were positively correlated to FFR-rFVIIa plasma levels ($r^2 = 0.82$; $p = 0.0001$).

Conclusion: TF pathway inhibition by FFR-rFVIIa significantly reduces platelet deposition in severely damaged vessels. This antithrombotic effect is accompanied by dose-dependent PT prolongation. Thus, TF inhibition appears to be a highly promising strategy for blocking atherothrombosis.

Key words: *: FFR-rFVIIa, tissue factor, factor VIIa, platelets, thrombosis, fibrin, platelet deposition.*

Effect of tissue factor pathway inhibition on platelet response to vascular damage
Sánchez S, Vilahur G, Casani L, Badimon L
Investigación Cardiovascular, 2003; 6: 42-53

Correspondencia / *correspondence:*
Prof. Lina Badimon
Centro de Investigación Cardiovascular
CSIC-Hospital Sta. Creu i S. Pau
C/ Jordi Girona, 18-26
08034 Barcelona
E-mail: lbmucv@cid.csic.es

INTRODUCCIÓN

Las manifestaciones clínicas de la enfermedad aterosclerótica coronaria están causadas mayoritariamente por una rotura de la placa seguida de la exposición a la circulación sanguínea de los componentes de su interior, que inducen la formación del trombo plaquetario (1-3). Se ha demostrado que el núcleo graso es el componente más trombogénico de la placa aterosclerótica (4); esta estructura tiene un gran contenido en factor tisular (TF), el cual parece predecir la trombogenicidad de la placa (5).

El TF es una glicoproteína unida a la membrana que, entre otras funciones, está implicada en la coagulación sanguínea. Mediante su unión al factor VIIa (FVIIa), el TF desencadena la cascada de la coagulación por la vía extrínseca, permitiendo la activación del factor X (FX) y promoviendo la formación de trombina (6). La implicación del TF en el proceso aterotrombótico ha sido previamente demostrada (5). De hecho, la inhibición local de este factor mediante el inhibidor de la vía del TF recombinante (*r*-TFPI) en placas ateroscleróticas humanas redujo su trombogenicidad (7). Por ello, la inhibición del TF parece una buena estrategia para reducir los episodios trombóticos asociados a la complicación aguda de las lesiones ateroscleróticas.

Recientemente se ha descrito un factor VIIa recombinante con el centro activo inhibido (FFR-rFVIIa) (8). Este compuesto inhibe competitivamente la unión del FVIIa (FIBA) al TF, interrumpiendo consecuentemente el proceso de coagulación mediado por este factor (vía del TF). Por lo tanto, el objetivo de este trabajo se ha centrado en demostrar que el FFR-rFVIIa al unirse al TF reducirá el riesgo trombótico asociado a las lesiones en la integridad de la arteria. Para ello, hemos utilizado la cámara de perfusión Badimon en condiciones hemodinámicas caracterizadas usando sustratos vasculares ligera y severamente dañados como desencadenantes del trombo.

INTRODUCTION

The clinical signs of coronary atherosclerotic disease are mainly the result of atheromaplaque rupture followed by exposure to the bloodstream of its components, which induce platelet thrombus formation (1-3). It has been shown that the fatty core is the most thrombogenic component of the atherosclerotic plaque (4). This structure has a high content in tissue factor (TF), which appears to predict plaque thrombogenicity (5).

TF is a membrane-bound glycoprotein involved in blood coagulation, among other functions. By binding to factor VIIa (FVIIa), TF triggers the coagulation cascade by the extrinsic pathway, allowing activation of factor X (FX) and promoting thrombin formation (6). Involvement of TF in atherothrombosis has been shown (5). In fact, local inhibition of this factor by recombinant TF pathway inhibitor (rTFPI) in human atherosclerotic plaques reduced its thrombogenicity (7). Thus, TF inhibition appears to be a good strategy for reducing thrombotic events associated with an acute complication of atherosclerotic lesions.

A recombinant active site inhibited factor VIIa (FFR-rFVIIa) has recently been reported (8). This compound competitively inhibits FVIIa (FIBA) binding to TF, thereby disrupting the coagulation process mediated by this factor (TF pathway). Therefore, the main objetive of this study was to show that, by binding to TF, FFR-rFVIIa will reduce the thrombotic risk associated with arterial damage. For this, the Badimon perfusion chamber was used under characterized hemodynamic conditions, using vascular substrates with mild or severe damage to trigger the thrombus.

ABREVIATURAS

TF: factor tisular.
FVII/FVIIa: factor VII/factor VII activado.
FX: factor X.
r-TFPI: inhibidor de la vía del factor tisular recombinante.
FFR-rFVIIa: factor VIIa recombinante con su centro activo inhibido.
PT: tiempo de protrombina.
aPTT: tiempo de la tromboplastina parcial activada.
SEM: error estándar.

ABBREVIATIONS

aPTT: activated partial thromboplastin time.
FFR-rFVIIa: recombinant active site inhibited factor VIIa.
FVII/FVIIa: factor VII/activated factor VII.
FX: factor X.
PT: prothrombin time.
rTFPI: recombinant tissue factor pathway inhibitor.
SEM: standard error of the mean.
TF: tissue factor.

MATERIALES Y MÉTODOS

Modelo experimental

El estudio se realizó usando como modelo experimental el cerdo. Los animales (cruce comercial, hembras, $n = 12$) se obtuvieron de una granja local (39,7 ± 3,3 kg) y se estabularon al menos durante siete días, en los que no se realizó ningún procedimiento experimental, en el estabulario del Laboratorio de Investigación Cardiovascular. El alojamiento se realizó individualmente, en un ambiente con temperatura (≈ 18 °C) y ciclo de luz día-noche (7 am–7 pm) controlados. El agua y el pienso se administró *ad libitum*.

Todos los procedimientos realizados en este estudio se realizaron de acuerdo con las guías institucionales y las guías para la investigación con animales de la American Heart Association.

Diseño experimental

Los parámetros hematológicos y de coagulación se determinaron en muestras sanguíneas obtenidas en condiciones basales y en distintos periodos de tiempo durante el proceso experimental. El día anterior a cada experimento, se extrajo una muestra sanguínea a partir de la cual se aislaron las plaquetas, se marcaron radiactivamente y fueron reinyectadas en el animal. Durante el proceso experimental se evaluó la deposición plaquetaria desencadenada por la pared vascular ligera y severamente lesionada, en condiciones de fuerza de cizalladura típicas de una arteria coronaria no estenótica (212 seg^{-1}) a diferentes tiempos de perfusión (cinco y diez minutos), utilizando la cámara Badimon.

Se ensayaron varias dosis de FFR-rFVIIa (cedido por Novo Nordisk, Dinamarca) (0,1, 0,5, 1 y 2 mg/kg) para evaluar el tiempo de protrombina (PT) y los niveles de fármaco en plasma así como su eficacia inhibiendo la formación del trombo. El tratamiento se realizó mediante un bolo lento intravenoso (i.v.). La deposición plaquetaria se evaluó antes y después de la administración del fármaco, de manera que cada animal fue su propio control. En estudios preliminares la dosis más baja (0,1 mg/kg) no tuvo efecto sobre la trombosis y la dosis superior (2 mg/kg) produjo un acusado alargamiento del PT, por ello se eligieron las dosis de 0,5 y 1 mg/kg para estudiar el efecto del FFR-rFVIIa sobre la trombosis.

MATERIAL AND METHODS

Experimental model

An experimental porcine model was used for the study. The animals (commercial breed; females, n = 12) were obtained from a local farm (body weight 39.7 ± 3.3 kg) and were housed for at least 7 days, in which no experimental procedures were performed, in the animal house of the Cardiovascular Research Center. The animals were housed individually at a temperature of ≈ 8 °C and under controlled light/dark conditions (7:00 am–19:00 pm). Food and water was provided ad libitum.

All procedures in this study were carried out in compliance with the corresponding institutional guidelines, and with the guidelines for animal research of the American Heart Association (AHA).

Experimental design

The hematological and coagulation parameters were measured in blood samples drawn at baseline and at different times during the experimental procedure. On the day before each experiment, a blood sample was drawn, from which platelets were isolated, radioactively labeled, and reinjected into the animal. During the experimental process, platelet deposition triggered by the mildly and severly damaged vascular wall was evaluated under the typical shear rate conditions in a non-stenotic coronary artery (212 sec^{-1}) at different perfusion times (five and ten minutes), using the Badimon chamber.

Several FFR-rFVIIa doses (supplied by Novo Nordisk, Denmark) were tested (0.1, 0.5, 1 and 2 mg/kg) to assess prothrombin time (PT) and plasma drug levels, as well as the efficacy of the drug for inhibiting thrombus formation. Treatment was carried out as a slow intravenous (i.v.) bolus. Platelet deposition was evaluated before and after drug administration, each animal thus acting as its own control. In preliminary studies, the lowest dose (0.1 mg/kg) had no effect upon thrombosis, while the highest dose (2 mg/kg) caused a marked PT prolongation. The 0.5 and 1 mg/kg doses were therefore selected to study the effect of FFR-rFVIIa upon thrombosis.

Parámetros de coagulación y hematológicos

A lo largo de todo el experimento se obtuvieron muestras sanguíneas seriadas en citrato trisódico (3,8%, 1:10), a partir de las cuales se realizó el recuento hematológico (System 9000, Serono) y la determinación de los siguientes parámetros de coagulación (coagulómetro ST4, Diagnostica Stago): PT, fibrinógeno y tiempo de la tromboplastina parcial activada (aPTT).

Coagulation and hematological parameters

Throughout the experimental study, serial blood samples were collected in trisodium citrate (3.8%, 1:10) and used for blood counts (System 9000, Serono) and to measure the following coagulation parameters (ST4 coagulometer, Diagnostica Stago): PT, fibrinogen and activated partial thromboplastin time (aPTT).

Determinación de los niveles plasmáticos de FFR-rFVIIa

Los niveles plasmáticos de FFR-rFVIIa se determinaron de manera secuencial y paralela a las determinaciones de PT, a lo largo del procedimiento experimental. El FFR-rFVIIa se determinó mediante un kit comercial de ELISA para determinar FVII humano (Dako Corp.), siguiendo la información prevista por la empresa proveedora. Para verificar que los valores obtenidos en plasma correspondían a FFR-rFVIIa, se realizaron determinaciones en muestras basales (previas al tratamiento) y se confirmó que sus niveles se encontraban por debajo del límite de detección.

Measurement of plasma FFR-rFVIIa levels

Plasma levels of FFR-rFVIIa were sequentially measured, in parallel to PT determinations, throughout the experimental procedure. FFR-rFVIIa was measured using a commercial ELISA kit for human FVII (Dako Corp.), following the manufacturer instructions. To verify that the values recorded in plasma corresponded to FFR-rFVIIa, measurements were made in baseline samples (i.e., prior to treatment), which confirmed that the levels were below the detection limit.

Agregación plaquetar *ex vivo*

Se realizaron estudios de agregación plaquetaria antes y después del tratamiento con FFR-rFVIIa. Se obtuvieron muestras sanguíneas en citrato sódico y se determinó la agregación en plasma rico en plaquetas (PRP) (agregómetro óptico, Aggrecorder II, Menarini) y en sangre total (agregómetro de impedancia, Chrono-Log) tras la adicción de agonistas plaquetarios (ADP, colágeno), siguiendo metodología previamente estandarizada (9).

Ex-vivo *platelet aggregation*

Platelet aggregation studies were made before and after treatment with FFR-rFVIIa. Blood samples were collected in sodium citrate and aggregation was measured in platelet-rich plasma (PRP) (Aggrecorder II optical aggregometer, Menarini) and in whole blood (Chrono-Log impedance aggregometer) after the addition of platelet agonists (ADP, collagen) according to previously standardized methods (9).

Marcaje de plaquetas

El día anterior al experimento se obtuvo una muestra sanguínea en ACD anticoagulante (ácido cítrico 0,04 M, citrato trisódico 0,09 M, dextrosa 0,07 M; 7:50), a partir de la cual se aislaron las plaquetas por centrifugación, que se marcaron con ^{111}In-oxina (Amersham) y finalmente se reinyectaron en el animal como se ha descrito previamente (10). La dosis de radioactividad inyectada fue de 296,8 ± 16,7 µCi, la lisis durante el marcaje de 1,0 ± 0,2 y la eficiencia del 89,4 ± 2,4%.

Platelet labeling

The day before the experiment, a blood sample was collected in anticoagulant ACD (citric acid 0.04 M, trisodium citrate 0.09 M, dextrose 0.07 M; 7:50), and centrifuged to isolate the platelets, that were labeled with ^{111}In-oxine (Amersham) and finally reinjected into the animal as previously described (10). The injected radioactivity dose was 296.8 ± 16.7 µCi, lysis during labeling was 1.0 ± 0.2, and the efficiency 89.4 ± 2.4%.

Experimento de trombosis

El día posterior al marcaje de plaquetas, el animal fue sedado (Azoperona intramuscular, 8 mg/kg, Stressnil, Esteve), anestesiado (tiobarbital sódico i.v., 10 mg/kg, B.BRAUM), intubado y heparinizado (heparina i.v., 50 U/kg/hora uhp, Liquemine, Roche). Se estableció un circuito extracorpóreo entre la vena yugular y la arteria carótida, en el que se colocó la cámara de perfusión Badimon, y se mantuvo una velocidad constante de flujo de 10 ml/min (Figura 1) (10-13). Se realizaron perfusiones de cinco y diez minutos a velocidades de cizalladura de 212 seg^{-1}, que corresponde al flujo

Thrombosis experiment

The day after platelet labeling, the animal was sedated (intramuscular azoperone, 8 mg/kg, Stressnil, Esteve), anesthetized (thiobarbital sodium, 10 mg/kg i.v., B. Braun), intubated and heparinized (heparin, 50 U/kg/hour i.v., Liquemine, Roche). An extracorporeal circuit was established between the jugular vein and carotid artery, in which the Badimon perfusion chamber was placed, maintaining a constant flow rate of 10 ml/min (Figure 1) (10-13). Perfusions were performed for five and ten minutes at shear

Figura 1. Representación esquemática de la cámara de perfusión y del circuito extracorpóreo en el cerdo. El sustrato vascular (10 x 30 mm) se coloca en la cámara de perfusión. La cámara se coloca en un baño atemperado a 37 °C y se conecta mediante tubos de silicona y polietileno a la arteria carótida y a la bomba peristáltica. La sangre sale succionada por la bomba a velocidad constante (10 ml/min) y pasa primero por la cámara; a continuación se recircula al animal por la vena yugular. Antes y después de perfundir la sangre se lava el circuito con PBS, el cual se desecha. Al finalizar la perfusión se extrae el sustrato vascular con cuidado y se determina la radiactividad.

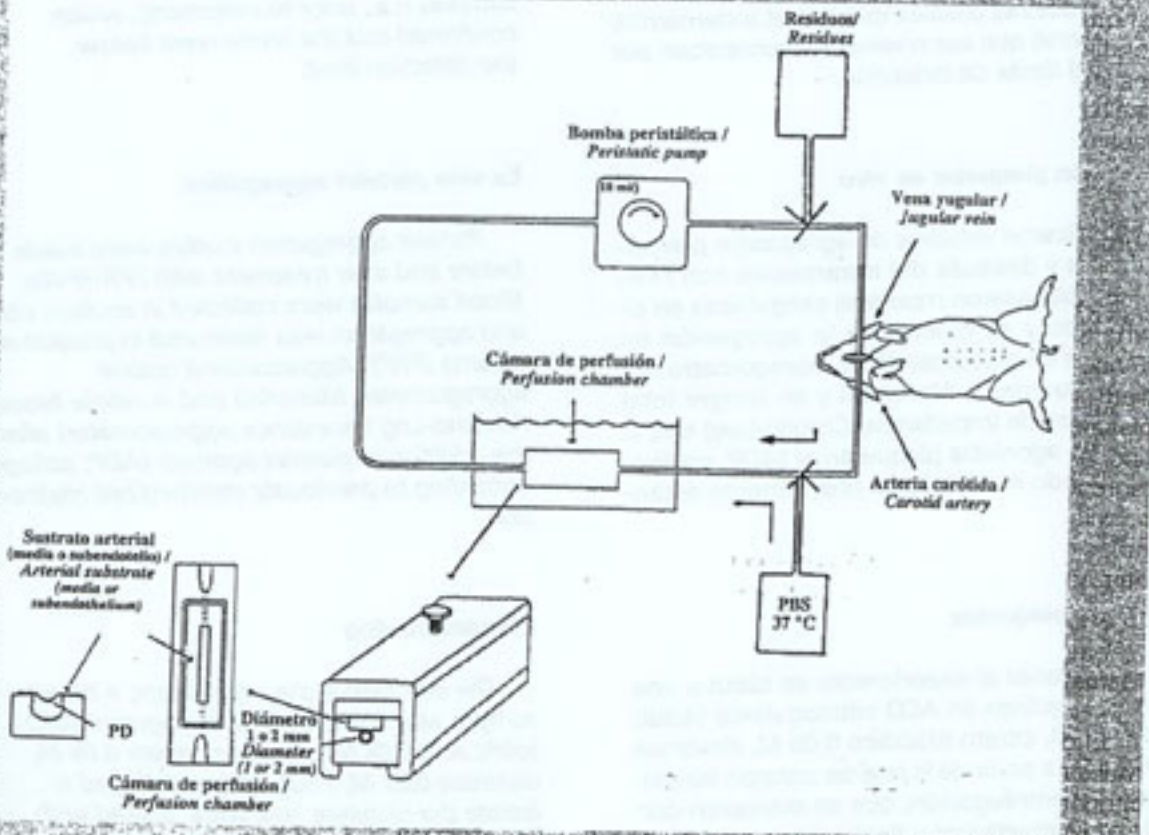

***Figure 1.** Schematic representation of the perfusion chamber and extracorporeal circuit in the pig. The vascular substrate (10 x 30 mm) is placed in the perfusion chamber, which is in turn immersed in a warm bath (37 °C) and connected by silicone and polyethylene tubes to the carotid artery and peristaltic pump. The blood is suctioned by the pump at a constant rate (10 ml/min) and first passes through the chamber. It then recirculates back into the animal through the jugular vein. Before and after blood perfusion, the circuit is flushed with phosphate buffered saline (PBS), which is then discarded. At the end of perfusion, the vascular substrate is carefully removed and its radioactivity is assessed.*

de las arterias coronarias no estenosadas (11). Como desencadenantes de la trombosis se utilizaron segmentos vasculares lesionados obtenidos a partir de aorta torácica porcina. Se expusieron dos tipos de lesión vascular al flujo sanguíneo: subendotelio, que simula lesiones ligeras o erosiones del endotelio (10); y túnica media, que simula lesiones arteriales severas como sucede cuando se rompe una placa aterosclerótica (10, 14). La radioactividad de los segmentos vasculares perfundidos se midió en un contador de radiación gamma (Wizard 1470, Wallac), y los resultados se expresaron como número de plaquetas depositadas por unidad de superficie (PLT's $x10^6/cm^2$) (10, 14). Al final del experimento, los animales fueron sometidos a eutanasia mediante una sobredosis de KCl (2 M i.v.).

Análisis estadísticos

Las comparaciones se realizaron con el programa de estadística STATVIEW II (Abacus Conceps Inc.). Los análisis entre varios grupos se llevaron a cabo utilizando el test no paramétrico de Kruskal Wallis, seguido del test Mann Witney para evaluar las diferencias específicas entre grupos. Los análisis para grupos apareados se realizaron con el test de Wilcoxon. Todos los valores se presentan como la media ± error estándar (SEM); valores de $p < 0,05$ se consideraron significativos.

RESULTADOS

Parámetros hematológicos y de coagulación

Los parámetros basales hematológicos y de coagulación no presentaron diferencias significativas entre grupos. El PT se alargó de manera significativa tras la administración de FFR-rFVIIa ($p = 0,0001$, Tabla I). Debido a que la vida media de este fármaco a las dosis utilizadas es corta, se realizó la determinación de los niveles de FFR-rFVIIa en plasma de manera secuencial y paralela al estudio del PT (Figura 2). Las determinaciones a partir de muestras basales estuvieron por debajo del límite de detección, confirmando que el test utilizado no reconocía el FVII/FVIIa endógeno. Los cambios obtenidos en el PT se correlacionaron muy significativamente y de manera positiva con los niveles de FFR-rFVIIa en plasma ($r^2 = 0,82$; $p = 0,0001$).

rates of 212 sec^{-1}, corresponding to the flow found in non-stenotic coronary arteries (11). Damaged vascular segments obtained from porcine thoracic aorta were used to trigger thrombosis. Two types of vascular damage were exposed to the blood flow: subendothelium, which simulates mild damage or erosions in the endothelium (10), and the media layer, which simulates severe arterial damage as occurs after rupture of an atherosclerotic plaque (10, 14). The radioactivity of the perfused vascular segments was measured using a gamma radiation counter (Wizard 1470. Wallac), and the results were expressed as the number of platelets deposited per surface unit (PLTs x $10^6/cm^2$) (10, 14). At the end of the experiment the animals were killed using an intravenous KCl overdose (2 M).

Statistical analysis

Comparisons were made using the Statview II statistical package (Abacus Concepts Inc.). Analyses between several groups were carried out using the Kruskal-Wallis nonparametric test, followed by the Mann-Whitney's U test for assessing specific differences between groups. Analyses of paired groups were performed with the Wilcoxon test. All values are expressed as the mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered significant.

RESULTS

Hematological and coagulation parameters

Baseline hematological and coagulation parameters showed no significant differences between groups. PT was significantly prolonged after administering FFR-rFVIIa ($p = 0.0001$; Table I). Since the half-life of this drug is short at the doses used, FFR-rFVIIa plasma levels were sequentially measured in parallel to the study of PT (Figure 2). The determinations in baseline samples were below the detection limit, confirming that the test used was unable to detect endogenous FFR-rFVIIa. The changes in PT obtained showed a high positive significant correlation to plasma FFR-rFVIIa levels ($r^2 = 0.82$; $p = 0.0001$).

TABLA I. Valores de PT en situación basal y tras la administración de FFR-rFVIIa / *TABLE I. PT values at baseline and after FFR-rFVIIa administration*

Grupo / *Group*	FFR-rFVIIa 0,1 mg/kg (*n* = 10)		FFR-rFVIIa 0,5 mg/kg (*n* = 9)		FFR-rFVIIa 1 mg/kg (*n* = 15)		FFR-rFVIIa 2 mg/kg (*n* = 3)		
Periodo	Basal / *Baseline*	Post-tto. / *Post-treat.*	Basal / *Baseline*	Post-tto. / *Post-treat.*	Basal / *Baseline*	Post-tto. / *Post-treat.*	Basal / *Baseline*	Post-tto. / *Post-treat.*	*Period*
PT (seg)	13.5 ± 0.2	17.8 ± 0.4*	14.3 ± 0.3	25.4 ± 0,5*†	13.7 ± 0.1	29.1 ± 0.4*†‡	14.2 ± 0.1	43.5 ± 0.7*†‡#	PT (sec)

Post-tto.: valores obtenidos tras la administración de FFR-rFVIIa.
Los resultados se expresan como media ± error estándar.
* $p < 0,05$ *vs* al valor basal; † $p < 0,05$ *vs* grupo tratado con la dosis de 0,1 mg/kg; ‡ $p < 0,05$ *vs* grupo tratado con la dosis de 0,5 mg/kg; # $p < 0,05$ *vs* grupo tratado con la dosis de 1 mg/kg.
Post-treatment: values obtained after administering FFR-rFVIIa.
The results are expressed as mean ± SEM.
* $p < 0.05$ vs *baseline*; † $p < 0.05$ vs *the group treated with the 0.1 mg/kg dose*; ‡ $p < 0.05$ vs *the group treated with the 0.5 mg/kg dose*; and # $p < 0.05$ vs *the group treated with the 1 mg/kg dose.*

Figura 2. Cinética del PT (A) y de los niveles plasmáticos de FFR-rFVIIa (B) a lo largo del procedimiento experimental de todas las dosis evaluadas. La dosis de 0 mg/kg incluye las determinaciones basales de todos los grupos.

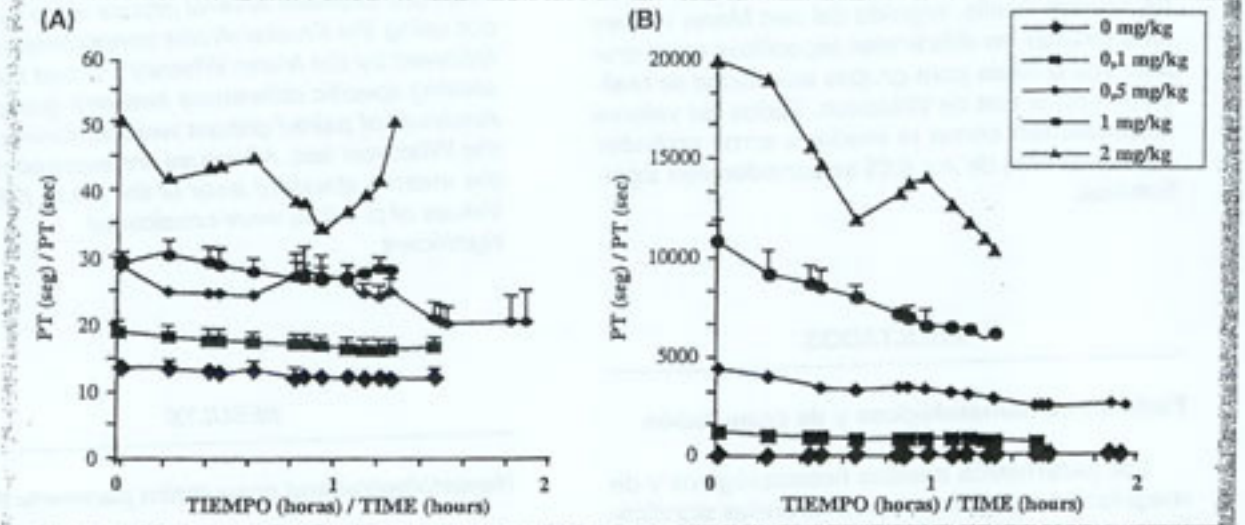

Figure 2. Kinetics of PT (A) and FFR-rFVIIa plasma levels (B) during the experimental procedure for all doses evaluated. The 0 mg/kg dose includes the baseline measurements for all groups.

A lo largo del procedimiento de trombosis los parámetros hematológicos se mantuvieron dentro de los intérvalos de normalidad para el cerdo y no se encontraron diferencias significativas entre grupos. El aPTT aumentó tras las heparinización (ratio 2,7 ± 0,1), pero ni en este parámetro ni en los niveles de fibrinógeno se vieron afectados por la administración de FFR-rFVIIa.

Throughout the thrombosis procedure, the hematological parameters remained within the normal range for swine, and no significant differences were found between groups. APTT increased following heparinization (ratio 2.7 ± 0.1), but neither this parameter nor fibrinogen levels were affected by FFR-rFVIIa administration.

Agregación plaquetar *ex vivo*

El tratamiento con FFR-rFVIIa no modificó la agregación plaquetaria *ex vivo* ni en sangre total ni en PRP, cuando se utilizaron colágeno y ADP como agonistas plaquetarios (los datos no se muestran).

Efectos del FFR-rFVIIa sobre la deposición plaquetaria

El FFR-rFVIIa no inhibió la deposición plaquetaria cuando se perfundió pared vascular ligeramente lesionada (control, $n = 7$; FFR-rFVIIa 0,5 mg/kg, $n = 3$; FFR-rFVIIa 1 mg/kg, $n = 4$) (Figura 3A). En cambio, la deposición plaquetaria desencadenada por pared vascular severamente lesionada fue inhibida significativamente ($p < 0,005$) tras la administración de FFR-rFVIIa (Figura 3B) (control, $n = 14$; FFR-rFVIIa 0,5 mg/kg, $n = 6$; FFR-rFVIIa 1 mg/kg, $n = 8$). El efecto inhibitorio máximo del FFR-rFVIIa se observó con la dosis de 0,5 mg/kg, no habiendo diferencias significativas respecto a la dosis de 1 mg/kg. Se puede apreciar al analizar la cinética de deposición sobre lesión severa a lo largo del tiempo, que con FFR-rFVIIa el crecimiento del trombo se frenó entre los cinco y los diez minutos, mientras en el grupo control el crecimiento fue constante (Figura 3B).

Ex-vivo *platelet aggregation*

Treatment with FFR-rFVIIa did not modify platelet aggregation ex vivo, in whole blood or in PRP, when collagen and ADP were used as platelet agonists (data not shown).

Effects of FFR-rFVIIa on platelet deposition

FFR-rFVIIa did not inhibit platelet deposition when mildly damaged vascular wall was perfused (control, n = 7; FFR-rFVIIa 0.5 mg/kg, n = 3; FFR-rFVIIa 1 mg/kg, n = 4)(Figure 3A). By contrast, platelet deposition triggered by severely damaged vascular wall was significantly inhibited ($p < 0.005$) after administering FFR-rFVIIa (control, n = 14; FFR-rFVIIa 0.5 mg/kg, n = 6; FFR-rFVIIa 1 mg/kg, n = 8)(Figure 3B). The maximum inhibitory effect of FFR-rFVIIa was seen with the 0.5 mg/kg dose, with no significant differences compared to the 1 mg/kg dose. When the deposition kinetics upon severe damage over time was analyzed, it was noted that FFR-rFVIIa arrested thrombus growth at between five and ten minutes, whereas constant growth occurred in the control group (Figure 3B).

Platelet deposition on severely damaged vascular wall showed a weak but statistically

Figura 3. Deposición plaquetar total sobre pared vascular con lesión ligera (A) y severa (B) perfundida a velocidad de cizalladura de 212 seg^{-1} durante cinco y diez minutos en los tres grupos de dosis. Los resultados de expresan como media ± error estándar. * $p < 0,005$ *vs* control.

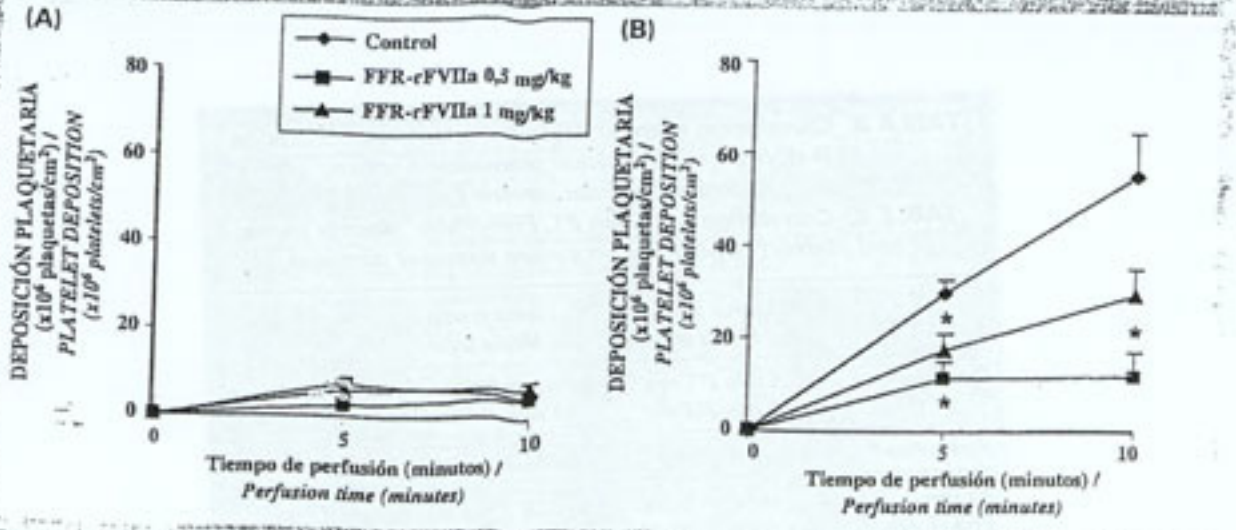

*Figura 3. Total platelet deposition on vascular wall with mild (A) and severe damage (B) perfused at a shear rate of 212 sec^{-1} for five and ten minutes, in the three dosage groups. The results are expressed as the mean ± SEM. * $p < 0.005$ vs control.*

La deposición plaquetaria sobre pared vascular severamente lesionada mostró una relación débil pero estadísticamente significativa con el PT y los niveles plasmáticos de FFR-rFVIIa (Tabla II).

DISCUSIÓN

Las manifestaciones clínicas de la enfermedad coronaria están causadas por la rotura de la placa aterosclerótica y, consecuente, exposición de su contenido a la sangre, dando lugar a la formación de un trombo que interrumpe de manera crónica o aguda el flujo sanguíneo (1-3). El tratamiento médico de elección en esta patología está dirigido a la prevención y disolución del trombo. Recientemente, ha sido propuesta una nueva estrategia terapéutica dirigida a inhibir la formación de trombina bloqueando la vía del TF. En el presente trabajo hemos investigado el efecto del FFR-rFVIIa, inhibidor competitivo del TF, sobre la trombosis desencadenada por lesiones de diverso grado de severidad.

Nuestros resultados demuestran que el FFR-rFVIIa es capaz de inhibir la deposición plaquetaria desencadenada por la exposición de lesión vascular severa (rotura de la pared vascular) a la circulación sanguínea. Por el contrario, el FFR-rFVIIa no tiene un efecto significativo en presencia de lesiones vasculares ligeras (erosión de la superficie vascular), lo que puede atribuirse a que los mecanismos que desencadenan la trombosis en estas condiciones no son dependientes de la vía del TF. Este último tipo de lesiones inducen un estí-

significant relationship to PT and plasma FFR-rFVIIa levels (Table II).

DISCUSSION

The clinical signs of coronary artery disease are due to rupture of the atherosclerotic plaque, with resultant exposure of the plaque contents to the bloodstream, which leads to formation of a thrombus that chronically or acutely interrupts blood flow (1-3). The medical treatment of choice in this condition is aimed at prevention of thrombus formation and its dissolution. A new therapeutic strategywith the aimed at inhibiting thrombin formation by blocking the TF pathway has recently been proposed. This study investigated the effect of FFR-rFVIIa, a competitive TF inhibitor, upon thrombosis triggered by vascular wall lesions of different degrees of severity.

The results obtained show that FFR-rFVIIa is able to inhibit platelet deposition triggered by exposure of severe vascular lesions (vascular wall rupture) to the bloodstream. By contrast, FFR-rFVIIa had no significant effect in the presence of mild vascular damage (erosion of the vessel surface), which may be attributed to the fact that the mechanisms which trigger thrombosis under these conditions are not dependent upon the TF pathway. Such mild vascular damage only induce a very weak thrombogenic stimulus, leading to platelet

TABLA II. Correlación entre el PT, los niveles plasmáticos de FFR-rFVIIa y la deposición plaquetaria sobre lesión vascular severa /
TABLE II. Correlation between PT, FFR-rFVIIa plasma levels, and platelet deposition on severe vascular damage

Condiciones /	Túnica media / *Media layer* 5 min 212 seg^{-1} / *5 min 212 sec^{-1}*		Túnica media / *Media layer* 10 min 212 seg^{-1} / *5 min 212 sec^{-1}*		*Conditions*
Parámetro /	r^2	p	r^2	p	*Parameter*
PT (seg)	0.24	0.008	0.26	0.007	*PT (sec)*
FFR-rFVIIa	0.21	0.013	0.114	0.08	*FFR-rFVIIa*

PT: tiempo de protrombina.
PT: prothrombin time.

mulo trombogénico muy poco intenso, dando lugar a la adhesión plaquetaria, mecanismo independiente de la cascada de la coagulación y de la deposición de fibrina (4,10). El trombo formado en estas condiciones es de un tamaño muy reducido y está formado principalmente por una monocapa de plaquetas adheridas a la superficie lesionada (11-15). La no participación del sistema de la coagulación, y por tanto de la vía del TF, en este mecanismo, explica que su bloqueo por FFR-rFVIIa no influya en el depósito de plaquetas. Por otro lado, las lesiones vasculares severas provocan un estímulo trombogénico mucho más potente, induciendo la activación plaquetaria acompañada de la activación de la vía del TF (6, 16, 17). El trombo formado, en este caso, es de un tamaño considerable y se compone de agregados plaquetarios con fibras de fibrina entrelazadas que le confieren estabilidad y lo anclan firmemente a la superficie vascular (4, 10, 12-15). Ya que la vía del TF da lugar a la formación de trombina, la cual es la principal responsable de la activación plaquetaria en situaciones de lesión vascular (18) así como de la transformación del fibrinógeno circulante en fibrina, la inhibición del TF puede suponer una estrategia relevante para la reducción del tamaño del trombo. De hecho, diversos estudios han descrito un efecto antitrombótico con diferentes tipos de inhibidores del TF (7, 19-26).

El bloqueo del TF por FFR-rFVIIa produce el alargamiento dosis-dependiente del PT, sin alteración del aPTT. La modificación del PT ha sido previamente descrita tras la administración sistémica de FFR-rFVIIa y otros inhibidores del TF en humanos (27, 28), conejos (20, 25, 26) y ratas (29). Además, la ligera correlación que hemos encontrado entre los niveles plasmáticos de FFR-rFVIIa, el PT y la deposición plaquetaria sobre lesión vascular severa, nos sugiere que probablemente hay una relación entre la vía del TF y la activación plaquetaria, como se afirma en varios trabajos (22).

El hecho de que la funcionalidad plaquetaria sea normal tras la adición de colágeno y ADP a plaquetas obtenidas de sangre anticoagulada de animales control y tratados demuestra que el efecto del FFR-rFVIIa es TF dependiente y no está implicado en otros efectos antiplaquetarios.

Hasta ahora, existen muy pocos estudios clínicos en humanos que evalúen inhibidores del TF, ya que son compuestos que todavía están en una fase de estudio bastante inicial. En un estudio en fase I para evaluar la farmacocinética, farmacodinámica y seguridad del FFR-rFVIIa administrado i.v. en voluntarios sanos, Erhardtsen *et al.* (28) observaron que este compuesto se toleraba muy bien a dosis entre 0,01 y 0,40 mg/kg, y que producía

adhesion, a mechanism independent from the coagulation cascade and fibrin deposition (4, 10). The thrombus formed under these conditions is of a very small size, and mainly consists of a monolayer of platelets adhered to the damaged surface (11-15).

The non-participation of the coagulation system, and thus of the TF pathway, in this mechanism explains why its blockage by FFR-rFVIIa does not influence platelet deposition. On the other hand, severe vascular damage induce a much more potent thrombogenic stimulus, inducing activation of both platelets and the TF pathway (6, 16, 17). The thrombus formed in this case is of a considerable size and consists of platelet aggregates with intermingling fibrin fibers that confer stability to the thrombus and anchor it firmly to the vascular surface (4, 10, 12-15). Since the TF pathway leads to the formation of fibrin, which is the main responsible for platelet activation in situations of vascular damage (18) and for the conversion of circulating fibrinogen into fibrin, TF inhibition may represent a relevant strategy for reducing thrombus size. In fact, various studies have reported an antithrombotic effect with different types of TF inhibitors (7, 19-26).

TF blockade by FFR-rFVIIa causes a dose-dependent prolongation of PT, with no changes in aPTT. PT changes have been previously reported after systemic administration of FFR-rFVIIa and other TF inhibitors in humans (27, 28), rabbits (20, 25, 26) and rats (29). Moreover, the minor correlation found in our study between FFR-rFVIIa plasma levels, PT and platelet deposition upon severe vascular lesions suggests that a relationship probably exists between the TF pathway and platelet activation, as suggested in several studies (22).

The fact that platelet function is normal after adding collagen and ADP to platelets obtained from anticoagulated blood collected of control and treated animals shows that the effect of FFR-rFVIIa is TF-dependent and is not involved in other antiplatelet effects.

To date, very few clinical studies evaluating TF inhibitors have been conducted in humans, since such compounds are still in quite early stages of research. In one phase I clinical trial to assess the pharmacokinetics, pharmacodynamics and safety of FFR-rFVIIa administered by the intravenous route in healthy volunteers, Erhardtsen et al. (28) found that this compound was very well tolerated at

una elongación dosis-dependiente del PT sin alterar el aPTT. Por otro lado, actualmente Lincoff *et al.* (30) están realizando actualmente un estudio en fase II para evaluar la eficacia y seguridad de FFR-rFVIIa en pacientes sometidos a angioplastia coronaria transluminal percútanea. Recientemente, un estudio clínico en fase II realizado con pacientes que padecían septicemia severa ha mostrado una tendencia a la reducción de la mortalidad a los 28 días en el grupo tratado con r-TFPI (31).

En resumen, este trabajo demuestra que el bloqueo de la vía del TF mediante FFR-rFVIIa reduce la trombosis desencadenada por lesión vascular severa y, paralelamente, produce un alargamiento del PT. Consecuentemente, se puede confirmar que el mecanismo de formación del trombo cuando existe una lesión vascular severa es dependiente del TF. Sería necesario realizar más estudios en este campo, especialmente en modelos ateroscleróticos, ya que la inhibición del TF podría representar una estrategia terapéutica a considerar en la prevención de la trombosis desencadenada por lesiones ateroscleróticas, donde la presencia del TF es muy relevante.

doses between 0.01 and 0.40 mg/kg, and caused a dose-dependent prolongation of PT without altering aPTT. On the other hand, Lincoff et al. *(30) are performing a phase II study to assess the efficacy and safety of FFR-rFVIIa in patients undergoing percutaneous transluminal coronary angioplasty (PTCA). Recently, a phase II clinical trial on patients with severe septicemia showed a trend to a reduced mortality at 28 days in the group treated with r-TFPI (31).*

To summarize, this study shows blockade of the TF pathway using FFR-rFVIIa reduces the thrombosis triggered by severe vascular damage, and induces a parallel prolongation of PT. Thus, it can be confirmed that the thrombus formation mechanism in the presence of severe vascular damage is TF-dependent. Further studies are required in this field, particularly in atherosclerotic models, since TF inhibition might offer a therapeutic strategy for preventing thrombosis triggered by atherosclerotic lesions, where the presence of TF is very relevant.

Agradecimientos

Este trabajo ha sido posible gracias a ayudas recibidas de PNS 2000/0174; Novo-Nordisk, Dinamarca y MAPFRE. Sonia Sánchez y Laura Casani son becarios predoctorales de la Fundación de Investigación Cardiovascular, y Gemma Vilahur es becario predoctoral BEFI.

Acknowledgements

Grants to perform this study were received from PNS 2000/0174; Novo-Nordisk, Denmark, and MAPFRE. Sonia Sánchez and Laura Casani are predoctorate scholarship holders of the Cardiovascular Research Foundation, and Gemma Vilahur predoctoral fellow BEFI.

BIBLIOGRAFÍA / REFERENCES

1. FUSTER V, BADIMON L, BADIMON J J, CHESEBRO J H. The pathogenesis of coronary artery disease and the acute coronary syndrome. *N Engl J Med.* 1992; 310-318.
2. BADIMON J J, FUSTER V, CHESEBRO J H, BADIMON L. Coronary atherosclerosis: a multifactorial disease. *Circulation.* 1993; 87 (suppl III): II-3-II16.
3. FALK E, SHAH P K, FUSTER V. Coronary plaque disruption. *Circulation.* 1995; 92: 657-671.
4. FERNANDEZ-ORTIZ A, BADIMON J J, FALK E, FUSTER V, MEYER B, MAILHAC A, WENG D, SHAH P K, BADIMON L. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. *J Am Coll Cardiol.* 1994; 23: 1462-1569.
5. TOSCHI V, GALLO R, LETTINO M, FALLON J T, GAAERTZ S D, FERNANDEZ-ORTIZ A, CHESEBRO J H, BADIMON L, NEMERSON Y, FUSTER V, BADIMON J J. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation.* 1997; 95: 594-599.
6. NEMERSON Y. Tissue factor and hemostasis. *Blood.* 1988; 71: 1-8.
7. BADIMON J J, LETTINO M, TOSCHI V, FUSTER V, BERROZPE M, CHESEBRO J, BADIMON L. Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques. *Circulation.* 1999; 99: 1780-1787.
8. SORENSEN B R, PERSSON E, FRESKGARD P O, KJALKE M, EZBAN M, WILLIAMS T, RAO L V M. Incorporation of an active site-inhibitor in factor VIIa alters the affinity for tissue factor. *J Biol Chem.* 1997; 272: 11863-11868.
9. GÁLVEZ A, BADIMON L, BADIMON JJ , FUSTER V. Electrical blood from human, pig and rabbit. *Thromb Haemost.* 1986; 56: 128-132.
10. BADIMON L, BADIMON J J, GÁLVEZ A, CHESEBRO J H, FUSTER V. Influence of arterial damage and wall

shear rate on platelet deposition. Ex vivo study in a swine model. *Arteriosclerosis.* 1986; 6: 312-320.
11. BADIMON L, TURITTO V T, ROSEMARK, BADIMON J J, FUSTER V. Characterization of tubular flow chamber for studying platelet interaction with biological and prosthetic materials. *J Lab Clin Med.* 1987; 6: 706-718.
12. BADIMON L, BADIMON J J, TURITTO V, VALLABHOJUSULA S, FUSTER V. Platelet thrombus formation on collagen type I: a model of deep vessel injury: influence of blood rheology, von Willebrand factor, and blood coagulation. *Circulation.* 1988; 78: 1431-1442.
13. BADIMON L, BADIMON J J. Mechanisms of arterial thrombosis in non parallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. *J Clin Invest.* 1989; 84: 1134-1144.
14. MAILHAC A, BADIMON J J, FERNÁNDEZ-ORTIZ A, MEYER B, CHESEBRO J H, FUSTER V, BADIMON L. Effect of an eccentric severe stenosis on fibrin(ogen) deposition on severely damaged vessel wall in arterial thrombosis. Relative contribution of fibrin(ogen) and platelets. *Circulation.* 1994; 90: 988-996.
15. LASSILA R, BADIMON J J, VALLBHAJOSULA S, BADIMON L. Dynamic monitoring of platelet deposition on severely damaged vessel wall in flowing blood. Effects of different stenosis on thrombus growth. *Arteriosclerosis.* 1990; 10: 306-315.
16. RAPAPORT S I, RAO L V M. The tissue factor pathway: how it has become a «prima ballerina». *Thrombo Haemost.* 1995; 74: 7-17.
17. FUSTER V, FALLON J T, NEMERSON Y. Coronary thrombosis. *Lancet.* 1996; 348 (suppl 1): s7.
18. KRISHNASWAMY S, FIELD K A, EDINGTON T S, MORRSSEY J H, MANN K G. Role of the membrane surface in the activation of human coagulation factor X. *J Biol Chem.* 1992; 267: 26110-26120.
19. KIRCHHOFER D, TSCHOPP T B, BAUMGARTNER H R. Active site-blocked factors VIIa and IXa and IX differentially inhibit fibrin formation in a human ex vivo thrombosis model. *Arterioscler Thromb Vasc Biol.* 1995; 15: 1098-1106.
20. JANG Y, GUZMAN L, LINCOFF M, GOTTSAUNER-WOLF M, FORUDI F, HART C H, COURTMAN D W, EZBAN M, ELLIS S G, TOPOL E J. Influence of blockade at specific levels of the coagulation cascade on restenosis in a rabbit model atherosclerotic femoral artery injury model. *Circulation.* 1995; 92: 3041-3050.
21. HARKER L, HANSON S, WILCOX J, KELLY A. Antithrombotic and antilesion benfits without hemorrhagic risks by inhibiting tissue factor pathway. *Haemostasis.* 1996; 26 (suppl 1): 76-82.
22. RAO L, EZBAN M. Active site-blocked activated factor VII as an effective antithrombotic agent: mechanism of action. *Blood Coagulation and Fibrinolysis.* 2000; 11 (suppl 1): S135-S143.
23. PAWASHE A B, GOLINO P, AMBROSIO G, MIGLIACCIO F, RAGNI M, PASCUCCI I, CHIARELLO M, BACH R, GAREN A, KONIGSBERG W K, *et al.* A monoclonal antibody against rabbit tissue factor inhibits thrombus formation in stenotic injured rabbit carotid arteries. *Circ Res.* 1994; 74 (1): 56-63.
24. GOLINO P, RAGNI M, CIRILLO P, D'ANDREA D, SCOGNAMIGLIO A, RAVERA A, BUONO C, EZBAN M, CORCIONE N, VIGORITO F, CONDORELLI M, CHIARELLO M. Antithrombotic effects of recombinant human, active site-blocked factor VIIa in a rabbit model of recurrent arterial thrombosis. *Circ Res.* 1998; 82: 39-46.
25. RAGNI M, GOLINO P, CIRILLO P, PASCUCCI I, SCOGNAMIGLIO A, RAVERA A, ESPOSITO N, BATTAGLIA C, GUARINO A, CHIARIELLO M. Inactivated factor VIIa exercices a poweful antithrombotic activity in an experimental model of recurrent arterial thrombosis. *Cardiologia.* 1996; 41 (1): 51-8.
26. ARNLJOTS B, SÖDERSTRÖM T, EZBAN M, HEDNER U. Effect of locally-applied active site-blocked activated factor VII (ASIS) on experimental arterial thrombosis. *Blood Coagul Fibrinolysis.* 2000, 11 (suppl 1): S145-S148.
27. KRISTENSEN A T, KRISTENSEN H I, EZBAN M. Active site inhibited recombinant factor VIIa (FFR-rFVIIa) dose depently inhibits thrombin generation in a whole vessel ex vivo model. *XVII Congress of the International Society of Thrombosis and Haemostasis.* Washington DC, USA, August 14-21, 1999 (abstract).
28. ERHARDSTEN E, NILSSON P, JOHANNNESSEN M, THOMSEN M. Pharmacokinetics and safety of FFR-rFVIIa after single doses in healthy subjects. *J Clin Pharmacol.* 2001; 41: 880-885.
29. HAN X, GIRARD T, BAUM P, ABENDSCHEIN D, BROZE G. Structural requirements for TFPI-mediated inhibition of neointimal thickening after ballon injury in the rat. *Arterioscler Thromb Vasc Biol.* 1999; 19: 2563-2567.
30. LINCOFF A M. First clinical investigation of a tissue-factor inhitor administered during percutaneous coronary revascularization: a randomized, double-blinded, dose-escalation trial: assesing safety and efficacy of FFR-rFVIIa in percutaneous transluminal coronary angioplasty (ASIS) trial. *J Am Coll Cardiol.* 2000; 36: 312-313 (abstract).
31. ABRAHAM E, REINHART K, SVOBODA P, SEIBERT A, OLTHOFF D, DAL NOGARE A, POSTIER R, HEMPELMANN G, BUTLER T, MARTIN E, ZWINGELSTEIN C, PERCELL S, SHU V, LEIGHTON A, CREASEY A A. Assessment of the safety recombinant tissue factor pathway inhibitor in patiens with severe sepsis: a multicenter, randomized, placebo-controlled, single-blind, dose escalation study. *Crit Care Med.* 2001; 29: 2081-2089.

European Heart Journal Supplements (2001) 3 (*Supplement I*), I16–I22

Atheromatous plaque formation and thrombogenesis: formation, risk factors and therapeutic approaches

L. Badimon, G. Vilahur, S. Sanchez and X. Duran

Cardiovascular Research Center, Instituto de Investigaciones Biomédicas de Barcelona and Consejo Superior de Investigaciones Cientificas, Hospital Santa Creu i San Pau-Universidad Autómoma de Barcelona, 08034 Barcelona, Spain

Angiographic and ultrasound analyses of the coronary arteries have confirmed the importance of acute thrombosis as the primary cause of myocardial infarction and acute coronary syndromes. Intravenous treatments aimed at recanalizing the obstructed arteries can help achieve acute reperfusion of the organ, but often a thrombus-triggering plaque remains active for some time. It is not yet known how long a plaque remains active, but it has been shown that systemic markers of coagulation remain elevated as long as 6 months after the event. The presence of a residual mural thrombus overlying an active plaque predisposes to recurrent thrombotic vessel occlusion. A fragmented thrombus appears to be one of the most powerful thrombogenic substrates, and residual thrombus may predispose to recurrent thrombosis. Non-acute, chronic antithrombotic treatments and pharmacological interventions should aim to block thrombosis and preserve vascular prostacyclin formation. Experimental work has shown that both aspirin and triflusal inhibit the growth of a thrombus on a fresh mural thrombus to the same extent, but triflusal was found to preserve cyclooxygenase-2 activity in the vessel wall. Research to uncover the mechanism of action of triflusal at the vascular level is in progress.

(Eur Heart J Supplements 2001; 3 (Suppl I): I16–I22

Key Words: Atherogenesis, lipids, platelets, thrombosis, triflusal.

Factors that favour the development of thrombi on atherosclerotic plaques

Thrombosis on atherosclerotic plaques produces the clinical manifestations of arterial disease[1,2]. Various components of atherosclerotic plaques have been shown to elicit specific types of thrombogenic activity. For example, lipid-rich plaques are up to six times as thrombogenic as all other types of plaque components[3]. The exact mechanisms of thrombogenicity of the lipid core are still under investigation. Lipid cores are rich in tissue factor (TF)[4], and this may account for some or most of their thrombogenicity. Thus, blocking tissue factor with a recombinant tissue factor pathway inhibitor has been shown to reduce the thrombogenicity of atherosclerotic plaques[5].

The origin of TF appears to be within macrophage-derived foam cells[6,7]. In specimens obtained during directional atherectomy, larger macrophage-rich areas were found in patients with unstable coronary syndromes than in patients with stable angina. Moreover, there was a significant correlation between these macrophage-rich areas and positive staining for TF in the atherectomy specimens from patients with unstable coronary syndromes[8–10]. Tissue factor may also derive from cell debris or microparticles released during apoptosis[11]. Plaque disruption may thus expose active TF in the plaque boundary layer to circulating blood, triggering acute thrombosis. Despite this evidence, TF in the lipid core may also be derived from sources other than monocytes, which have yet to be fully investigated.

An additional pool of TF has been found in the blood stream; however, its origin has yet to be determined and, although endothelial cells, macrophages, smooth muscle cells and myocardial cells may be the source of circulating TF, it may also derive from blood monocytes, neutrophils, or both. Tissue factor antigen has been detected in membrane vesicles that cluster near the surface of platelets[12]. In addition, monocytes and neutrophils rich in TF have been identified in peripheral blood, and the search for sources of TF continues to be an area of active research.

There is substantial experimental and clinical evidence that a primary hypercoagulable or thrombogenic state in the blood stream can promote focal thrombus formation[1,2]. This is of particular importance when considering the risk of complicating thrombosis after plaque rupture, and confirms that factors in addition to the atherosclerotic plaque are of great importance in

Correspondence: Professor Lina Badimon, Centro de Investigación y Desarrollo-CSIC, Jordi Girona, 18-26, 08034 Barcelona, Spain.

1520-765X/01/010016+07 $35.00/0

assessing thrombotic risk. Systemic factors, including alterations in lipid and hormonal metabolism, haemostasis, fibrinolysis, and platelet and leukocyte function, are known to be associated with increased blood reactivity and thrombogenicity. Increased plasma levels of catecholamines may favour platelet reactivity. Platelet aggregation and the generation of thrombin by circulating catecholalmines have been documented experimentally[13–15], and this association probably helps to explain the link between emotional stress[16] and circadian variation (i.e. early morning clustering of events with myocardial infarction)[17–19]. There is increasing evidence of enhanced platelet reactivity in cigarette smokers[20–22]. The association between enhanced thrombogenicity and smoking is further confirmed by the sharp decline in acute vascular events, most often associated with thrombosis, when smoking ceases[23,24].

Hypercholesterolaemia has been linked with hypercoagulability and enhanced platelet reactivity in numerous studies[25–32]. Young patients with a strong family history of coronary artery disease seem to have increased platelet reactivity. It should be noted that the hypercoagulable state associated with hypercholesterolaemia can be reversed if lipid levels are normalized with lipid-lowering therapy[28–30,33,34].

Among the new risk factors for arterial thrombosis and for atherosclerosis is homocysteine. This substance increases TF activity in endothelial cells [possibly in conjunction with lipoprotein(a)], and inhibits the expression of endothelial cell surface thrombomodulin (the substance central to the activation of protein C) and the binding activity of antithrombin III to endothelial heparin sulfate. Homocysteine, through these various mechanisms, seems to reduce the natural anticoagulant properties of the normal endothelium[35,36].

Abnormalities in the fibrinolytic pathways lead to increased thrombogenic risk in patients with coronary artery disease[37–39]. A correlation between high levels of plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator and crosslinked fibrin with the progression of atherosclerotic disease has been documented. Furthermore, it has been shown that plasma levels of fibrinogen, von Willebrand factor (vWF) and tissue-type plasminogen activator in patients with angina pectoris are independent predictors of subsequent myocardial infarction or sudden death[26]. High levels of PAI-1 correlated with the cholesterol levels in patients with some types of dyslipidamia. Although this suggests a potential mechanism by which hypercholesterolaemia is associated with increased thrombogenicity, the association of PAI-1 with coronary artery disease and acute myocardial infarction is unclear, and reports published so far are contradictory[26].

Other haemostatic proteins have also been investigated with regard to their role as thrombogenic risk factors. Several prospective studies have concluded that high plasma fibrinogen concentrations are independent risk factors for coronary artery disease and myocardial infarction[40]. The mechanism by which fibrinogen contributes to atherogenesis is not well understood; hypotheses include increased fibrin formation, increased viscosity, platelet aggregation and stimulation of smooth muscle cell proliferation. Fibrinogen levels correlate with age, degree of obesity, hyperlipidaemia, diabetes, smoking and emotional stress — all conditions that are themselves associated with atherosclerosis.

Coronary vasospasm may play an important role in the pathogenesis of acute coronary syndromes, as documented in electrocardiographic and angiographic studies[41]. In minor plaque disruption with a small thrombotic response, vasoactive substances can still be released by both the platelet and the arterial wall, further compromising coronary blood flow. Coronary artery vasospasm was found to be an important contributor to the phenomenon of intermittent coronary artery occlusion in patients with acute myocardial infarction.

Clinical manifestations of atherosclerotic plaques

The clinical manifestations of atherosclerotic plaques depend on several factors, including the degree and abruptness of blood flow obstruction, duration of decreased myocardial perfusion, myocardial oxygen demand at the time of the blood flow obstruction, and the extent of the thrombotic response to plaque disruption. Plaque disruption (whether by erosion or rupture) may be accompanied by haemorrhage into the plaque and a variable amount of luminal thrombosis. If the thrombus is small, plaque disruption probably proceeds unnoticed. If the thrombus is large enough to compromise blood flow through the coronary artery, however, the individual may experience an acute ischaemic syndrome.

Disruption of an atherosclerotic plaque in the coronary arteries plays a fundamental role in the development of the acute coronary syndromes (including unstable angina pectoris, acute myocardial infarction or sudden cardiac death)[1,2]. Angioscopic studies have documented the presence of intraluminal thrombi in both unstable angina[42–46] and acute myocardial infarction[47,48].

It is likely that when injury to the vessel wall is mild, the thrombogenic stimulus is relatively limited and the resulting thrombotic occlusion is transient, as occurs in unstable angina[1,2]. However, deep vessel injury, as seen with plaque rupture, results in the exposure of collagen, lipids and other intravascular components, leading to more persistent thrombotic occlusion and acute myocardial infarction[49]. Unstable angina, non-Q-wave and Q-wave myocardial infarction represent a continuum of the same disease process and, in contrast to stable angina, are usually characterized by an abrupt reduction in coronary artery blood flow[49]. In unstable angina, occlusion of the thrombotic vessel tends to be transient and episodic, leading to anginal symptoms at rest.

In addition to the effects of plaque disruption, other mechanisms may contribute to the reduction in coronary flow. As mentioned earlier, platelets that have attached to the disrupted plaque release vasoactive substances, including thromboxane A_2 and serotonin, which promote the aggregation of more platelets in the area and thus induce vasoconstriction. Alterations in perfusion probably account for 60–70% of all cases of unstable angina. The remainder appear to be mainly due to transient increases in myocardial oxygen demand[50].

In non-Q-wave myocardial infarction, the angiographic morphology of the culprit lesion is similar to that seen in unstable angina, confirming that plaque disruption is common to both syndromes. However, about 25% of the patients with non-Q-wave myocardial infarction have a totally occluded infarct-related artery on early angiography, with the distal myocardium supplied by collateral vessels[49]. The presence of ST-segment elevation in the electrocardiogram, an early peak in plasma creatine kinase concentration, and a high rate of angiographic patency of the infarct-related artery, all suggest that complete coronary occlusion followed by early reperfusion (<2 h) due to partial or total resolution of the thrombus, vasospasm or both, are important in the pathogenesis of most non-Q-wave myocardial infarctions. Thus, limiting the duration of myocardial ischaemia, spontaneous lysis of the thrombus, resolution of the vasospasm, or a well-developed collateral circulation, can prevent the formation of Q-wave myocardial infarction[49].

Deep arterial injury or ulceration results in the formation of a fixed, persistent thrombus leading to the abrupt cessation of myocardial perfusion and necrosis of Q-wave myocardial infarction[1,2]. The coronary artery lesion responsible for the infarction is frequently only mildly to moderately stenotic, suggesting that plaque rupture with subsequent thrombosis rather than the severity of the underlying lesion is the primary source of the occlusion[51]. Although a single severe stenosis can occlude more frequently than less severe stenoses, lesser stenoses account for more coronary artery occlusions because they are more frequent[52]. Furthermore, the less severe stenoses are much less likely to be associated with protective collateral circulation, and thus occlusion is more likely to lead to an acute clinical event[53]. In approximately 25% of patients with Q-wave infarction, coronary thrombosis results from superficial intimal injury in association with high-grade stenosis[1,2].

The acute onset of malignant ventricular dysrhythmias (ventricular tachycardia and ventricular fibrillation) appears to account for the syndrome of sudden cardiac death[54]. However, two distinct mechanisms play a role in the pathogenesis of these dysrhythmias. First, in patients with a substrate for the generation and maintenance of malignant ventricular dysrhythmias (such as extensive myocardial infarction or cardiomyopathy), an episode of such dysrhythmia can lead to sudden cardiac death[54]. Second, a rapidly progressing coronary artery lesion, in which plaque rupture and subsequent thrombosis lead to acute myocardial hypoperfusion in the absence of collateral flow, may also induce malignant ventricular dysrhythmias and sudden cardiac death[54].

Platelet activation and agonists of platelet activity

Exposed matrix from the vessel wall and thrombin generated by the activation of the coagulation cascade, along with circulating agonists, may be powerful platelet activators[55]. Most platelet aggregation agonists seem to act through the hydrolysis of platelet membrane phosphatidylinositol by phospholipase C, which results in the mobilization of free calcium from the platelet-dense tubular system. Adenosine diphosphate (ADP) is a platelet agonist that may be released from haemolysed red cells in the area of vessel injury. Each agonist stimulates the discharge of calcium from the platelet-dense tubular system and promotes the contraction of the platelet, with the subsequent release of its granular contents. Platelet-released ADP and serotonin stimulate adjacent platelets, further enhancing the process of platelet activation. Arachidonate, which is released from the platelet membrane by the stimulatory effect of collagen, thrombin, ADP and serotonin, is another platelet agonist. Arachidonate is converted to thromboxane A_2 by the sequential effects of cyclooxygenase and thromboxane synthase. Thromboxane A_2 not only promotes further platelet aggregation but is also a potent vasoconstrictor. Platelet receptors for thrombin, epinephrine, thromboxane A_2, and platelet-activating factor have been cloned and shown to resemble other G protein-coupled receptors, with a characteristic structure comprised of a single polypeptide with seven transmembrane domains.

The signal transduction mechanisms initiated upon binding of agonists to membrane-spanning receptors on the platelet surface have been partially elucidated. Agonist binding triggers cascades of intracellular second messengers, including inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate releases Ca^{2+} from the platelet-dense tubular system, raising the cytosolic concentration of free Ca^{2+}. Diacylglycerol activates protein kinase C, a serine/threonine kinase, translocating it to the plasma membrane and triggering granule secretion and fibrinogen receptor exposure (glycoprotein IIb/IIIa complex). At the same time, the rising concentration of cytosolic free Ca^{2+} facilitates arachidonate release from phospholipids by phospholipase A_2, a process that may occur at both the plasma membrane and the dense tubular system membrane. Arachidonate is metabolized to thromboxane A_2, which diffuses out of the cell, interacts with receptors on the platelet surface, and causes further platelet activation.

The initial recognition of vessel wall damage by platelets involves: (a) adhesion, activation and adherence to recognition sites on the thromboactive substrate (extracellular matrix proteins, e.g. vWF, collagen,

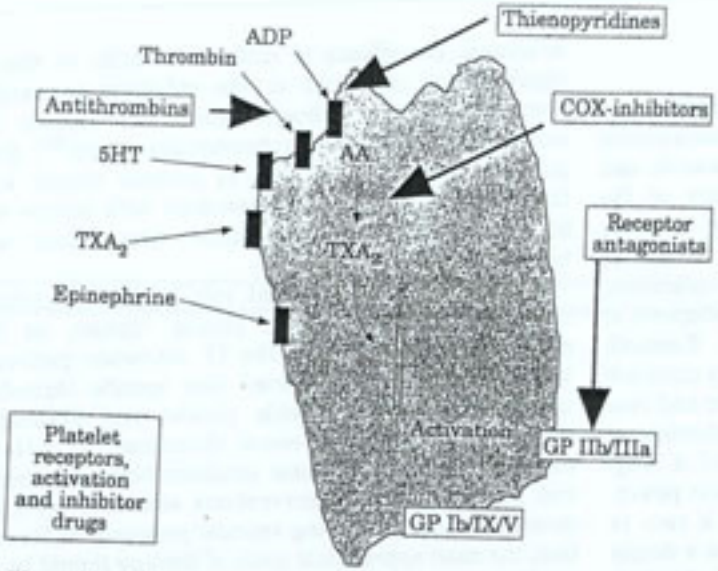

Figure 1 **Schematic diagram of platelet receptors and activation pathways (thin arrows). Classes of drugs that block receptors or pathways are shown (thick arrows).**

fibronectin, vitronectin, laminin); (b) spread of the platelets on the surface; and (c) aggregation with each other to form a platelet plug or white thrombus. The efficiency of platelet recruitment will depend on the underlying substrate and local geometry. In the final step of thrombus formation other blood cells are also recruited, and erythrocytes, neutrophils and occasionally monocytes can thus be found on evolving mixed thrombi.

Platelet function depends on adhesive interactions, and most of the glycoproteins on the platelet membrane surface are receptors for adhesive proteins. Many of these receptors have been identified, cloned, sequenced and classified within large families of genes that mediate a variety of cellular interactions. The most abundant is the integrin family, which includes GPIIb/IIIa, GPIa/IIa, GPIc/IIa, the fibronectin receptor and the vitronectin receptor, in decreasing order of magnitude. Another gene family present in the platelet membrane glycocalyx is the leucine-rich glycoprotein family represented by the GPIb/IX complex, a receptor for vWF on unstimulated platelets that mediates adhesion to the subendothelium and glycoprotein V[55–58].

Randomly distributed on the surface of resting platelets are about 50 000 molecules of GPIIb/IIIa. The complex is composed of one molecule of GPIIb (disulfide-linked heavy and light chains) and one of GPIIIa (single polypeptide chain). It is a Ca^{2+}-dependent heterodimer non-covalently associated on the platelet membrane[59]. Calcium is required for maintenance of the complex and for binding of adhesive proteins. On activated platelets, GPIIb/IIIa is a receptor for fibrinogen, fibronectin, vWF, vitronectin and thrombospondin[60]. The receptor recognition sequences are localized to small peptide sequences (Arg-Gly-Asp [RGD]) in the adhesive proteins[61]. Fibrinogen contains two RGD sequences in its α-chain, one near the N-terminus (residues 95–97) and a second near the C-terminus (residues 572–574)[62]. Fibrinogen has a second recognition site for GPIIb/IIIa, the 12-amino-acid sequence located at the carboxyl-terminus of the γ-chain of the molecule. This dodecapeptide is specific for fibrinogen and does not contain the RGD sequence, but competes with RGD-containing peptides for binding to GPIIb/IIIa.

Thrombin, one of the most potent known agonists for platelet activation and recruitment, plays an important role in the pathogenesis of arterial thrombosis. The thrombin receptor has 425 amino acids with seven transmembrane domains and a large NH_2-terminal extracellular extension that is cleaved by thrombin to produce a 'tethered' ligand that activates the receptor to initiate signal transduction[63,64]. Thrombin is a critical enzyme in early thrombus formation, cleaving fibrinopeptide A and B from fibrinogen to yield insoluble fibrin which effectively anchors the evolving thrombus. Both free and fibrin-bound thrombin are able to convert fibrinogen to fibrin, allowing propagation of the thrombus at the site of injury.

Therefore, platelet activation triggers intracellular signalling and the expression of platelet membrane receptors for adhesion and initiation of cell contractile processes that will induce shape changes and secretion of the granular contents. The expression of integrin IIb/IIa (aIIbb3) receptors for adhesive glycoprotein ligands (mainly fibrinogen and vWF) in the circulation initiate platelet-to-platelet interaction. The process is perpetuated by the arrival of platelets from the circulation. Most of the glycoproteins in the platelet membrane surface are receptors for adhesive proteins or mediate cellular interactions (Fig. 1).

Sustained activation

Residual thrombi on atherosclerotic plaques elicit sustained thrombotic activity, as shown by measurements of biological markers of the coagulation cascade and platelet activity. However, spontaneous lysis of the thrombus can occur in unstable angina and in acute myocardial infarction[65,66]. In these patients, as well as in those undergoing thrombolysis for acute infarction, the presence of a residual mural thrombus predisposes to recurrent thrombotic vessel occlusion[67–70]. Research has shown that a residual mural thrombus may encroach into the vessel lumen, increasing the shear rate and thus facilitating the activation and deposition of platelets on the lesion[71,72]. Additionally, the surface of a fragmented thrombus appears to be one of the most powerful thrombogenic stimuli. Platelet deposition is two- to fourfold as great on a residual thrombus as on a deeply injured arterial wall[68]. In a canine model of coronary thrombolysis, reocclusion of a recanalized artery was related mainly to high local thrombin activity on the surface of the fragmented thrombus[73].

Blockade of secondary thrombus growth on a preformed residual mural thrombus is an important therapeutic target to prevent reischaemia and reinfarction. The pharmacological approach to preventing secondary thrombus growth requires chronic oral treatment with minor or no side-effects; however, we still do not know from a pathophysiological standpoint how long treatment should last. Thrombin seems to be one of the primary agonists of platelets in thrombus growth on preformed thrombi[45]. After lysis or fragmentation of an ongoing thrombus, thrombin may be exposed to the circulating blood, leading to platelet activation and deposition, activation of coagulation and further thrombosis[74,75]. Finally, aside from active thrombin from the clot itself, recent studies have suggested that platelet or thrombin activity is also enhanced by the thrombolytic agents themselves.

Triflusal, an inhibitor of platelet aggregation

Triflusal [2-(acetyloxy)-4-(trifluoromethyl)benzoic acid], a platelet aggregation inhibitor structurally related to acetylsalicylic acid, inhibits thromboxane A_2 formation by irreversibly inhibiting platelet cyclooxygenase-1 activity. At therapeutic doses it has negligible or very weak effects on prostacyclin biosynthesis[76]. In addition, triflusal is an inhibitor of cAMP phosphodiesterase, and is capable of increasing platelet levels of cAMP[77]. Its main metabolite in plasma, 3-hydroxy-4-(trifluoromethyl)benzoic acid, is more potent than triflusal as an inhibitor of cAMP-phosphodiesterase, and has a longer half-life (34·3 h)[78–80]. At therapeutic doses, the risk of haemorrhage associated with triflusal is very low[81,82].

The Triflusal in Myocardial Infarction (TIM) study[82] showed that, in the acute phase of myocardial infarction, the efficacy of triflusal is similar to that of aspirin when measured as the reduction in cardiac events. Moreover, triflusal significantly reduces the incidence of non-fatal cerebrovascular events[82]. Subgroup analyses showed that, in patients treated with thrombolysis, aspirin was associated with higher risk of haemorrhagic cerebrovascular events than was triflusal[83].

Thrombin plays a pivotal role in the thrombotic response to atherosclerotic plaque rupture, as the enzyme is formed through the TF activation pathway. We have previously reported that specific thrombin inhibition markedly curtails platelet and fibrinogen deposition onto a fresh mural thrombus[5,74,75]. However, for non-acute, chronic antithrombotic treatments and pharmacological interventions aimed at blocking thrombosis and preserving vascular prostacyclin formation, the most appropriate goals of therapy should be to ameliorate the continuous prothrombotic triggering process and foment local vascular homeostasis. In an experimental animal model we have tested the efficacy of aspirin and triflusal in inhibiting secondary thrombus formation and protecting vascular cyclooxygenase. Both drugs inhibit the growth of a thrombus on a fresh mural thrombus to the same extent, but triflusal was found to preserve cyclooxygenase-2 activity in the vessel wall[84]. Research to uncover the mechanism of action of triflusal at the vascular level is in progress.

References

[1] Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (Part I). N Engl J Med 1992; 326: 242–50.

[2] Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (Part II). N Engl J Med 1992; 326: 310–8.

[3] Fernandez-Ortiz A, Badimon JJ, Falk E *et al.* Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 1994; 23: 1562–9.

[4] Toschi V, Gallo R, Lettino M *et al.* Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. Circulation 1997; 95: 594–9.

[5] Badimon JJ, Lettino M, Toschi V *et al.* Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques. Effects of TFPI on plaque thrombogenicity under flow conditions. Circulation 1999; 99: 1780–7.

[6] Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 1989; 86: 2839–43.

[7] Thiruvikraman SV, Guha A, Roboz J *et al.* In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. Lab Invest 1996; 75: 451–61.

[8] Annex BH, Denning SM, Channon KM *et al.* Differential expression of tissue factor protein in directional atherectomy specimens from patients with stable and unstable coronary syndromes. Circulation 1995; 91: 619–22.

[9] Moreno PR, Falk E, Palacios IF *et al.* Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation 1994; 90: 775–8.

[10] Moreno PR, Bernardi VH, Lopez-Cuellar J *et al.* Macrophages, smooth muscle cells, and tissue factor in unstable

angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes. Circulation 1996; 94: 3090–7.
[11] Mallat Z, Hugel B, Ohan J *et al.* Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999; 99: 348–53.
[12] Giesen PL, Rauch U, Bohrmann B *et al.* Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci USA 1999; 96: 2311–5.
[13] Badimon L, Martinez-Gonzalez J, Royo T, Lassila R, Badimon JJ. A sudden increase in plasma epinephrine levels transiently enhances platelet deposition on severely damaged arterial wall. Thromb Haemost 1999; 82: 1736–42.
[14] Spalding A, Vaitkevicius H, Dill S *et al.* Mechanism of epinephrine-induced platelet aggregation. Hypertension 1998; 31: 603–7.
[15] Goto S, Handa S, Takahashi E *et al.* Synergistic effect of epinephrine and shearing on platelet activation. Thromb Res 1996; 84: 351–9.
[16] Krantz DS, Kop WJ, Santiago HT, Gottdiener JS. Mental stress as a trigger of myocardial ischemia and infarction. Cardiol Clin 1996; 14: 271–87.
[17] Muller JE, Stone PH, Turi ZG *et al.* Circadian variation in the frequency of onset of acute myocardial infarction. N Engl J Med 1985; 313: 1315–22.
[18] Johnstone MT, Mittleman M, Tofler G, Muller JE. The pathophysiology of the onset of morning cardiovascular events. Am J Hypertens 1996; 9: 22S–28S.
[19] Willich SN, Linderer T, Wegscheider K *et al.* Increased morning incidence of myocardial infarction in the ISAM Study: absence with prior beta-adrenergic blockade. ISAM Study Group. Circulation 1989; 80: 853–8.
[20] Winniford MD, Wheelan KR, Kremers MS *et al.* Smoking-induced coronary vasoconstriction in patients with atherosclerotic coronary artery disease: evidence for adrenergically mediated alterations in coronary artery tone. Circulation 1986; 73: 662–7.
[21] Fuster V, Chesebro JH, Frye RL, Elveback LR. Platelet survival and the development of coronary artery disease in the young adult: effects of cigarette smoking, strong family history and medical therapy. Circulation 1981; 63: 546–51.
[22] Blann AD, Kirkpatrick U, Devine C *et al.* The influence of acute smoking on leucocytes, platelets and the endothelium. Atherosclerosis 1998; 141: 133–9.
[23] Paul O. Background of the prevention of cardiovascular disease. II. Arteriosclerosis, hypertension, and selected risk factors. Circulation 1989; 80: 206–14.
[24] Buhler FR, Vesanen K, Watters JT, Bolli P. Impact of smoking on heart attacks, strokes, blood pressure control, drug dose, and quality of life aspects in the International Prospective Primary Prevention Study in Hypertension. Am Heart J 1988; 115: 282–8.
[25] Hunt BJ. The relation between abnormal hemostatic function and the progression of coronary disease. Curr Opin Cardiol 1990; 5: 758–65.
[26] Thompson SG, Kienast J, Pyke SD *et al.* Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N Engl J Med 1995; 332: 635–41.
[27] Pueyo Palazón C, Alfón J, Gaffney P, Barrozpe M, Royo T, Badimon L. Effects of reducing LDL and increasing HDL with Gemfibrozil in experimental coronary lesion development and thrombotic risk. Atherosclerosis 1998; 136: 333–45.
[28] Alfón J, Pueyo-Palazón C, Royo T, Badimon L. Effects of statins in thrombosis and aortic lesion development in a dyslipemic rabbit model. Thromb Haemost 1999; 81: 822–7.
[29] Alfón J, Royo T, García-Moll X, Badimon L. Platelet deposition on eroded vessel wall at stenotic shear rate is inhibited by lipid-lowering treatment with atorvastatin. Arterioscler Thromb Vasc Biol 1999; 19: 1812–7.
[30] Alfón J, Guasch JF, Berrozpe M, Badimon L. NOSII gene expression correlates with atherosclerotic intimal thickening. Preventive effects of HMG-CoA reductase inhibitors. Atherosclerosis 1999; 145: 325–31.
[31] Badimon JJ, Badimon L, Turitto VT, Fuster V. Platelet deposition at high shear rates is enhanced by high plasma cholesterol levels. In vivo study in the rabbit model. Arterioscler Thromb 1991; 11: 395–402.
[32] Henry PD, Cabello OA, Chen CH. Hypercholesterolemia and endothelial dysfunction. Curr Opin Lipidol 1995; 6: 190–5.
[33] Lacoste L, Lam JY, Hung J *et al.* Hyperlipidemia and coronary disease. Correction of the increased thrombogenic potential with cholesterol reduction. Circulation 1995; 92: 3172–7.
[34] Rauch U, Osende JI, Chesebro JH *et al.* Statins and cardiovascular diseases: the multiple effects of lipid-lowering therapy by statins. Atherosclerosis 2000; 153: 181–9.
[35] Boers GH. Hyperhomocysteinemia as a risk factor for arterial and venous disease. A review of evidence and relevance. Thromb Haemost 1997; 78: 520–2.
[36] de Jong SC, van den Berg M, Rauwerda JA, Stehouwer CD. Hyperhomocysteinemia and atherothrombotic disease. Semin Thromb Hemost 1998; 24: 381–5.
[37] Vaughan DE. Plasminogen activator inhibitor-1: a common denominator in cardiovascular disease. J Invest Med 1998; 46: 370–6.
[38] Geppert A, Graf S, Beckmann R *et al.* Concentration of endogenous tPA antigen in coronary artery disease: relation to thrombotic events, aspirin treatment, hyperlipidemia, and multivessel disease. Arterioscler Thromb Vasc Biol 1998; 18: 1634–42.
[39] Salomaa V, Stinson V, Kark JD *et al.* Association of fibrinolytic parameters with early atherosclerosis. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation 1995; 91: 284–90.
[40] Meade TW. Fibrinogen and cardiovascular disease. J Clin Pathol 1997; 50: 13–5.
[41] Maseri A, L'Abbate A, Baroldi G *et al.* Coronary vasospasm as a possible cause of myocardial infarction. A conclusion derived from the study of 'preinfarction' angina. N Engl J Med 1978; 299: 1271–7.
[42] de Feyter PJ, Ozaki Y, Baptista J *et al.* Ischemia-related lesion characteristics in patients with stable or unstable angina. A study with intracoronary angioscopy and ultrasound. Circulation 1995; 92: 1408–13.
[43] Silva JA, Escobar A, Collins TJ *et al.* Unstable angina. A comparison of angioscopic findings between diabetic and nondiabetic patients. Circulation 1995; 92: 1731–6.
[44] Sherman CT, Litvack F, Grundfest W *et al.* Coronary angioscopy in patients with unstable angina pectoris. N Engl J Med 1986; 315: 913–9.
[45] Mizuno K, Satomura K, Miyamoto A *et al.* Angioscopic evaluation of coronary-artery thrombi in acute coronary syndromes. N Engl J Med 1992; 326: 287–91.
[46] Nesto RW, Waxman S, Mittleman MA *et al.* Angioscopy of culprit coronary lesions in unstable angina pectoris and correlation of clinical presentation with plaque morphology. Am J Cardiol 1998; 81: 225–8.
[47] Van Belle E, Lablanche JM, Bauters C *et al.* Coronary angioscopic findings in the infarct-related vessel within 1 month of acute myocardial infarction: natural history and the effect of thrombolysis. Circulation 1998; 97: 26–33.
[48] Uchida Y, Tomaru T, Nakamura F *et al.* Percutaneous coronary angioscopy in patients with ischemic heart disease. Am Heart J 1987; 114: 1216–22.
[49] Theroux P, Fuster V. Acute coronary syndromes: unstable angina and non-Q-wave myocardial infarction. Circulation 1998; 97: 1195–206.
[50] Braunwald E, Jones RH, Mark DB *et al.* Diagnosing and managing unstable angina. Agency for Health Care Policy and Research. Circulation 1994; 90: 613–22.
[51] Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation 1995; 92: 657–71.

[52] Alderman EL, Corley SD, Fisher LD *et al.* Five-year angiographic follow-up of factors associated with progression of coronary artery disease in the Coronary Artery Surgery Study (CASS). CASS Participating Investigators and Staff. J Am Coll Cardiol 1993; 22: 1141–54.
[53] Danchin N. Is myocardial revascularisation for tight coronary stenoses always necessary? Lancet 1993; 342: 224–5.
[54] Mehta D, Curwin J, Gomes JA, Fuster V. Sudden death in coronary artery disease: acute ischemia versus myocardial substrate. Circulation 1997; 96: 3215–23.
[55] Badimon L, Badimon JJ, Fuster V. Pathogenesis of thrombosis. In: Verstraete M, Fuster V, Topol, eds. Cardiovascular thrombosis: Thrombocardiology. Philadelphia: Lippincott-Raven, 1998: 23–44.
[56] Kieffer N, Phillips DR. Platelet membrane glycoproteins: functions in cellular interactions. Annu Rev Biol 1990; 6: 329–57.
[57] Kunicki TJ. Organization of glycoproteins within the platelet plasma membrane. In: George JN, Nurden AT, Philips DR, eds. Platelet membrane glycoproteins. New York: Plenum Press, 1985: 87–101.
[58] Marcus A, Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. FASEB J 1993; 7: 516–22.
[59] Fitzgerald LA, Phillips DR. Calcium regulation of the platelet membrane glycoprotein IIb–IIIa complex. J Biol Chem 1985; 260: 11366–76.
[60] Plow EF, Ginsberg MH, Marguerie GA. Expression and function of adhesive proteins on the platelet surface. In: Phillips DR, Shuman MA, eds. Biochemistry of platelets. New York: Academic Press, 1986: 225–56.
[61] Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion : RGD and integrins. Science 1987; 238: 491–7.
[62] Doolittle RF, Watt KWK, Cottrell BA, Strong DD, Riley M. The amino acid sequence of the a-chain of human fibrinogen. Nature (London) 1979; 280: 464–7.
[63] Vu TH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991; 64: 1057–68.
[64] Coughlin SR. Thrombin receptor structure and function. Thromb Haemost 1993; 70: 184–7.
[65] Rentrop KP, Feit F, Blanke H, Sherman W, Thornton JC. Serial angiographic assessment of coronary artery obstruction and collateral flow in acute myocardial infarction. Circulation 1989; 80: 1166–75.
[66] Van de Werf F, Arnold AER and the European Cooperative Study Group for Recombinant Tissue-Type Plasminogen Activator (rt-PA). Effect of intravenous tissue plasminogen activator on infarct size, left ventricular function and survival in patients with acute myocardial infarction. Br Med J 1988; 297: 374–9.
[67] Van Lierde, De Geest H, Verstraete M *et al.* Angiographic assessment of the infarct-related residual coronary stenosis after spontaneous or therapeutic thrombolysis. J Am Coll Cardiol 1990; 16: 1545–9.
[68] Fuster V, Stein B, Badimon L, Badimon JJ, Ambrose JA, Chesebro JH. Atherosclerotic plaque rupture and thrombosis: evolving concepts. Circulation 1990; 82 (Suppl II): 47–59.
[69] Davies SW, Marchant B, Lyon JP *et al.* Coronary lesion morphology in acute myocardial infarction: demonstration of early remodeling after streptokinase treatment. J Am Coll Cardiol 1990; 16: 1079–86.
[70] Gulba DC, Barthels M, Westhoff-Bleck M *et al.* Increased thrombin levels during acute myocardial infarction. Relevance for the success of therapy. Circulation 1991; 83: 937–44.
[71] Badimon L, Badimon JJ. Mechanism of arterial thrombosis in non-parallel strealines: platelet grow at the apex of stenotic severely injured vessel wall. Experimental study in the pig model. J Clin Invest 1989; 84: 1134–44.
[72] Lassila R, Badimon JJ, Vallabhajosula S, Badimon L. Dynamic monitoring of platelet deposition on severely damaged vessel wall in flowing blood. Effects of different stenosis on thrombus growth. Arteriosclerosis 1990; 10: 306–15.
[73] Fitzgerald DJ, Fitzgerald GA. Role of thrombin and thromboxane A_2 in reocclusion following coronary thrombolysis with tissue-type plasminogen activator. Proc Natl Acad Sci USA 1989; 86: 7585.
[74] Meyer BJ, Badimon JJ, Mailhac A *et al.* Inhibition of growth of thrombus on fresh mural thrombus: targeting optimal therapy. Circulation 1994; 90: 2432–8.
[75] Meyer B, Badimon JJ, Chesebro JH, Fallon JT, Fuster V, Badimon L. Dissolution of mural thrombus by specific thrombin inhibition with r-hirudin: comparison with heparin and aspirin. Circulation 1998; 97: 681–5.
[76] De la Cruz JP, Pavia J, García-Arnes J *et al.* Effects of triflusal and acetylsalicylic acid on platelet aggregation in whole blood of diabetic patients. Eur J Haematol 1998; 40: 232–6.
[77] García-Rafanell J, Ramis J, Gómez L *et al.* Effect of triflusal and other salicylic acid derivatives on cyclic AMP levels in rat platelets. Arch Int Pharmacodyn Ther 1986; 284: 155–65.
[78] Ramis J, Mis R, Forn J, Torrent J, Gorina E, Jané F. Pharmacokinetics of triflusal and its main metabolite HTB in healthy subjects following a single oral dose. Eur J Drug Metab Pharmacokinet 1991; 16: 269–73.
[79] Ramis J, Torrent J, Mis R *et al.* Pharmacokinetics of triflusal after single and repeated doses in man. Int J Clin Pharmacol Ther Toxicol 1990; 28: 344–9.
[80] Mis R, Ramis J, Conte L, Forn J. In vitro protein binding interaction between a metabolite of triflusal, 2-hydroxy-4-trifluoromethylbenzoic acid and other drugs. J Pharm Pharmacol 1992; 44: 935–7.
[81] Putz Ph, Buyes H, Delvaux D *et al.* Triflusal versus acetylsalicylic acid: a double-blind study for the prophylaxis of deep vein thrombosis after hip surgery. Acta Chir Belg 1991; 91: 269–76.
[82] Cruz-Fernández JM, López-Bescós L, García-Dorado D *et al.* Randomized comparative trial of triflusal and aspirin following acute myocardial infarction. Eur Heart J 2000; 21: 457–65.
[83] López-Bescós L, Calades O'Callaghan A, Castro Beiras A *et al.* Incidence of vascular stroke in patients with acute myocardial infarction receiving fibrinolytic treatment. Eur Heart J Suppl 1999; 1 (Suppl F): F19–F23.
[84] Sanchez-Gomez S, Alfón J, Badimon L. Thrombus growth on residual thrombus is inhibited and vascular wall Cox2 upregulated by treatment with Triflusal. Eur Heart J (Abstract Suppl) 2000; 21: 79 p546.