VNIVERSITAT DE VALÈNCIA

Facultad de Ciencias Biológicas
Doctorado en Bioquímica y Biomedicina

Estudio funcional de polimorfismos de la vía de activación de la proteína C

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Valencia, mayo de 2017

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AGRADECIMIENTOS

En primer lugar quisiera agradecer al Dr. España por la oportunidad que me brindó para iniciar mi carrera en la investigación, por entender mis inquietudes, dirigirme y acogerme en el grupo haciéndome sentir parte de él siempre.

También agradecer a la Dra. Medina por su ayuda en la codirección de la tesis, por su apoyo constante y entusiasmo que transmite a todos. Y por supuesto, agradecer a la Dra. Navarro, mi gran mentora en este proyecto que empezó con los cordones, continuó con muchas batallas, profesionales y personales, y aunque ahora concluya con esta tesis, otra parte permanecerá.

A mis compañeros del laboratorio, en especial a Laura, que ha sido mi media naranja en los muchos momentos vividos.

A mis amigos Silvia y Juancar, mi familia en Valencia.

A Huelva, y sobre todo a su gente.

Y por supuesto a mi familia, en especial a mi madre y mi hermana, sus ánimos nunca me han faltado. Gràcies.

Esta tesis es el producto de la ilusión que todos ellos me transmiten a mí.

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1. INTRODUCCIÓN

1.1. Sistema hemostático

La hemostasia es el conjunto de procesos que mantienen la sangre en estado fluido, encargándose de realizar dos misiones diferentes pero complementarias. Por un lado, detecta las roturas producidas en el árbol vascular y detiene las pérdidas de sangre que se pudieran producir y, por otro, mantiene la permeabilidad de los vasos. Esto requiere que la sangre pase a veces del estado fluido al sólido, y de nuevo al fluido, pero únicamente en el sitio de la rotura del vaso y en el momento y en la extensión adecuados. De ello se encarga la hemostasia, la cual envuelve un elevado número de reacciones e interacciones complejas entre los componentes sanguíneos y la pared de los vasos (Maruyama I, 1998).

Cuando un vaso sanguíneo se rompe o daña, se ponen en marcha los mecanismos de la hemostasia, que podemos agrupar en cuatro fases:

A) La hemostasia primaria, que comprende:

1) La contracción de la pared del vaso

2) La adhesión de las plaquetas a la zona de la pared dañada y agregación de las plaquetas entre sí.

B) La hemostasia secundaria:

3) La formación y consolidación del coágulo de fibrina (coagulación)

4) La eliminación del coágulo (fibrinólisis)

Todos estos mecanismos son esenciales para una hemostasia normal, y están perfectamente sincronizados y relacionados entre sí mediante un balance hemostático perfecto. La alteración de alguno de estos mecanismos puede romper esta sincronía, bien a favor de la coagulación y formación de un trombo dando lugar a una trombosis o bien a favor de frenar la formación del coágulo y acelerar su disolución dando lugar a una hemorragia.

Hasta hace algunos años se creía que la pared del vaso sanguíneo era una estructura inerte que actuaba de mero contenedor de la sangre. Sin embargo, hoy sabemos que la pared del vaso participa activamente en la hemostasia. La pared del vaso contiene tres

partes bien diferenciadas, la íntima, la capa media y la adventicia (Figura 1). La íntima es la capa más interna, formada por una monocapa de células endoteliales o endotelio vascular, en continuo contacto con la sangre circulante, y juega un papel fundamental en la hemostasia. Las células del endotelio vascular sintetizan y liberan multitud de sustancias importantes no solo para la hemostasia, sino también para otros procesos como la inflamación, la arteriosclerosis y el crecimiento.

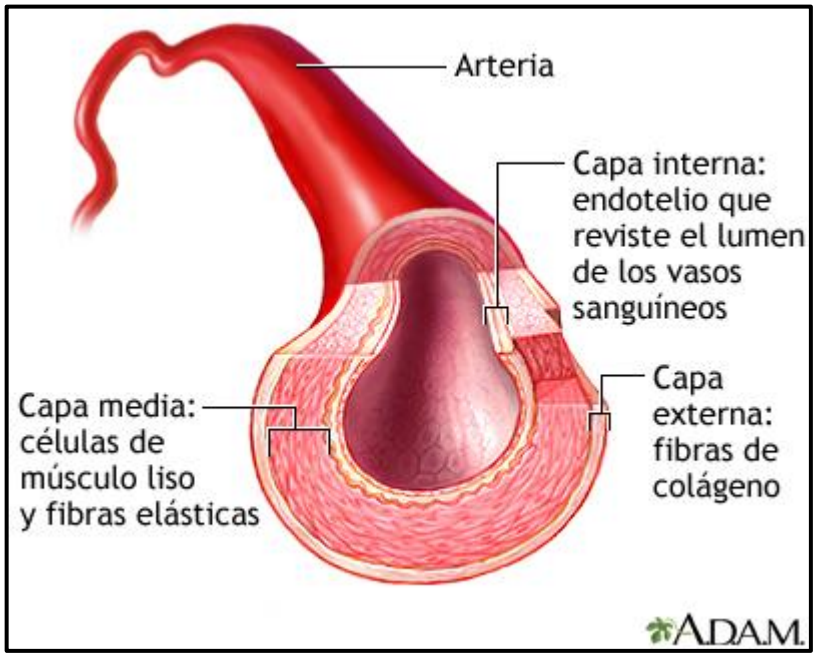

Figura 1. Estructura de la pared de un vaso sanguíneo

Con respecto a la hemostasia, el endotelio vascular sintetiza y libera multitud de sustancias, unas con actividad antitrombótica y otras protrombóticas (Figura 2). Así, el endotelio sintetiza o segrega sustancias antitrombóticas, tanto antiagregantes como la prostaciclina, anticoagulantes como la trombomodulina (TM), la proteína S (PS), el receptor endotelial de la proteína C (EPCR) y los glicosaminoglicanos, o profibrinolíticas como los activadores del plasminógeno de tipo tisular (tPA) o uroquinasa (uPA) y los receptores (R) de los activadores de la fibrinólisis: tPA-R y uPA-R. También sintetiza o segrega factores protrombóticos, tanto proagregantes como el factor von Willebrand (FVW), la fibronectina, la trombospondina, el factor activante plaquetario y el fibrinógeno, o procoagulantes como el factor tisular (FT), fibrinógeno y los factores (F) V, VIII y XI, o antifibrinolíticos como los inhibidores del activador del plasminógeno

(PAI) tipos 1, 2 y 3, el receptor de la fibrinolisis dirigido al PAI-1, y la capacidad de activar al inhibidor de la fibrinólisis activable por trombina (TAFI). En condiciones normales, la balanza está inclinada hacia la actividad antitrombótica, y el endotelio actúa como un eficiente antitrombótico. Pero cuando el crítico balance se rompe hacia uno u otro lado, puede sobrevenir una trombosis o una hemorragia.

Figura 2. Sustancias sintetizadas y/o liberadas por el endotelio vascular

Una vez iniciada la hemostasia primaria, las plaquetas liberan también sustancias procoagulantes. Además, aunque la plaqueta activada puede exponer FT, la rotura del vaso por sí misma expone al torrente circulatorio una gran cantidad de FT, que inicia la hemostasia secundaria. Todo ello hace que la hemostasia secundaria se inicie casi simultáneamente a la primaria. A efectos didácticos se suele describir como un sistema formado por cuatro partes (Figura 3): la coagulación y la anticoagulación, que controlan la formación del coágulo sanguíneo, y la fibrinolisis y la antifibrinolisis, que controlan la eliminación del coágulo. Tanto las respuestas como las actividades de las cuatro partes están coordinadas por la trombina. La trombina modifica las plaquetas para acelerar la coagulación. También modifica las células endoteliales para inhibir la coagulación y para

liberar PAI, activando la fibrinolisis. La trombina convierte al fibrinógeno en fibrina, la cual se polimeriza para formar el coágulo. Además, la trombina activa al FXIII, el cual estabiliza a la fibrina, haciéndola resistente a su disolución. Así, la respuesta del sistema hemostático (formación y disolución del coágulo) es dependiente de la concentración de trombina y de la normalidad de las cuatro dianas sobre las que actúa la trombina (plaquetas, células endoteliales, fibrinógeno y FXIII). Si las plaquetas son anormales, pueden no responder a la trombina y la coagulación se frena, originando hemorragias. Si las células endoteliales son anormales, pueden no responder a la trombina y resultar en trombosis.

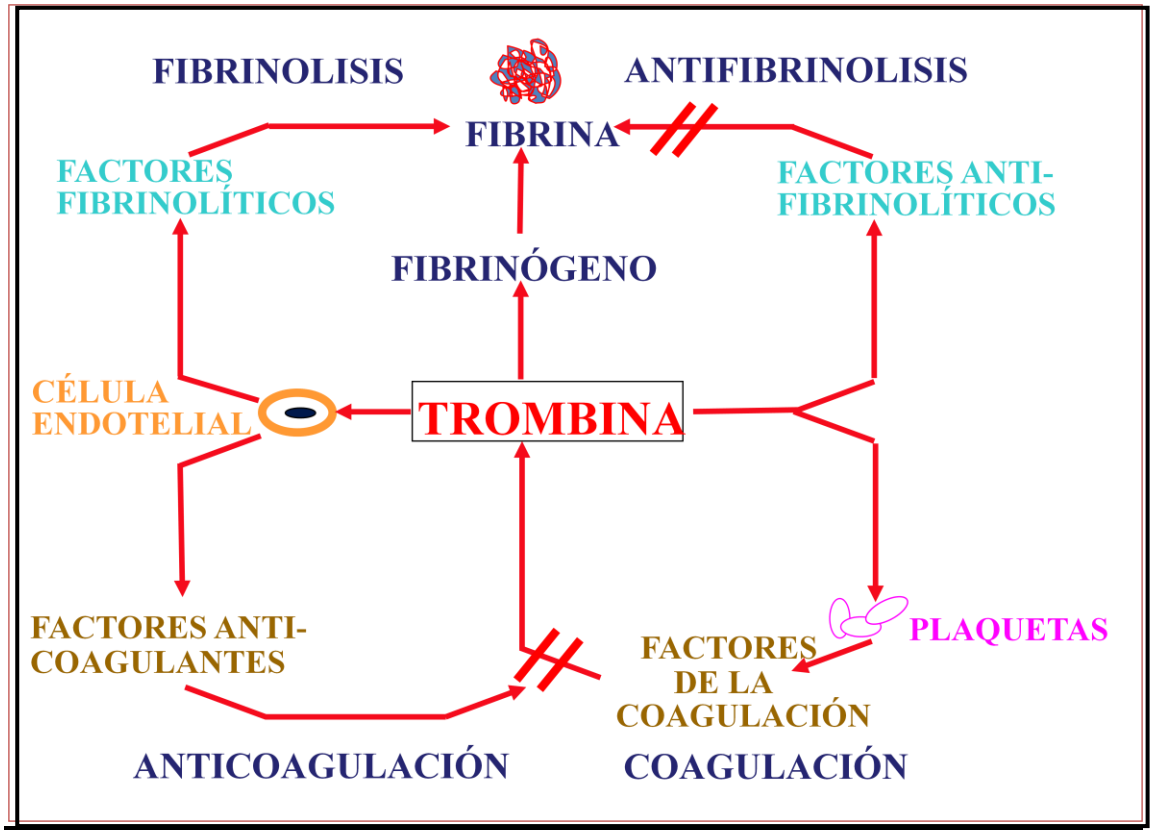

Figura 3. Esquema de la hemostasia secundaria

En el clásico mecanismo de la coagulación, ésta se define como una cascada proteolítica que puede iniciarse por dos vías enzimáticas convergentes, llamadas vía extrínseca y vía intrínseca (Figura 4). *In vivo*, la principal vía de activación de la coagulación es la vía extrínseca. Esta vía se inicia cuando el FT se expone en la superficie del vaso dañado y entra en contacto con trazas circulantes de FVII activado (FVIIa). El

complejo FT-FVIIa activa al FX iniciando la cascada que finalmente forma el coágulo de fibrina.

La trombina es proteína central del proceso de coagulación. Este proceso es relativamente lento en sus etapas iniciales. Pero nada más formarse las primeras trazas de trombina, ésta activa mecanismos de retroalimentación positivos, que estimulan la propia formación de trombina.

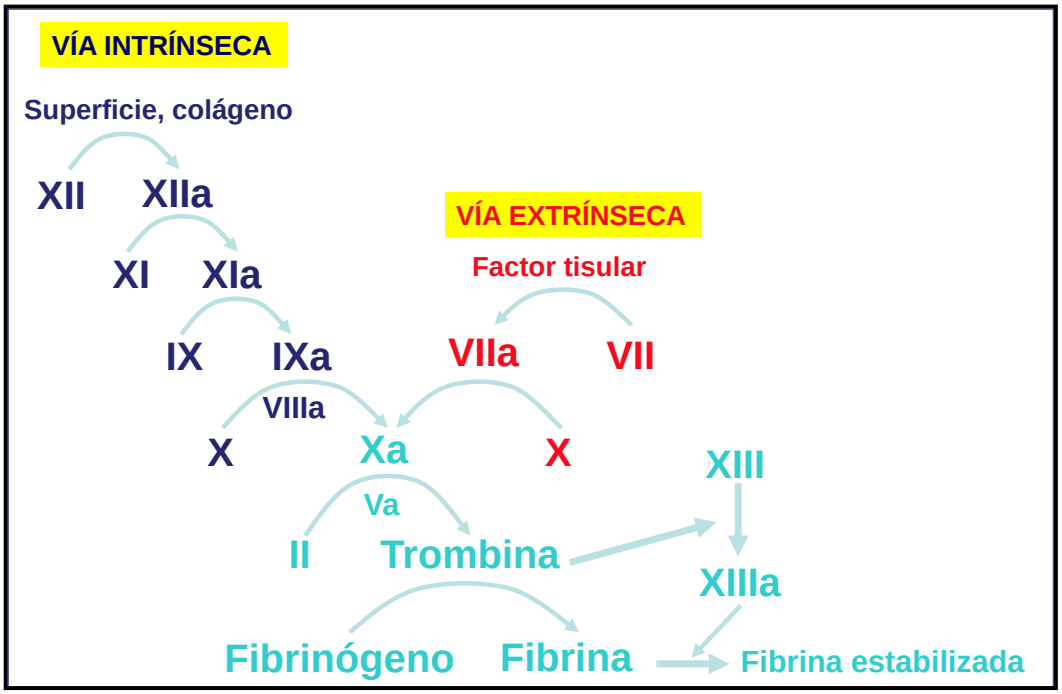

Figura 4. Esquema clásico de la cascada de la coagulación sanguínea.

1.2. Mecanismos de regulación de la coagulación sanguínea

La propia naturaleza de la coagulación sanguínea exige mecanismos de regulación que eviten su propagación y extensión de un modo incontrolado. De hecho, la trombosis es la principal causa de mortalidad y morbilidad en países desarrollados, puesto que puede dar lugar a infarto de miocardio, accidente cerebrovascular y trombosis venosa (Reddy KS, 2004). Por ello, resulta de gran interés conocer los mecanismos que regulan la formación del trombo. El requerimiento de una adecuada superficie cargada negativamente limita considerablemente su propagación más allá del lugar del daño vascular, al restringir las reacciones enzimáticas en dicho lugar. Otro mecanismo de

control es la fibrinolisis, que actúa desde un primer momento limitando el crecimiento del trombo. Sin embargo, existe un control directo que limita la actividad de los enzimas de la coagulación, denominado mecanismo de la anticoagulación. El mecanismo de anticoagulación está formado por tres sistemas inhibitorios complementarios: la antitrombina (AT), el inhibidor de la vía del factor tisular (TFPI) y el sistema de la proteína C (PC) (Figura 5). La AT inhibe a la mayoría de los factores de la coagulación activados, mientras que el TFPI inhibe la formación de FXa por el complejo FT/FVIIa. El sistema de la PC se encarga de inhibir a los cofactores de la coagulación Va y VIIIa, que son esenciales para la formación del FXa y trombina.

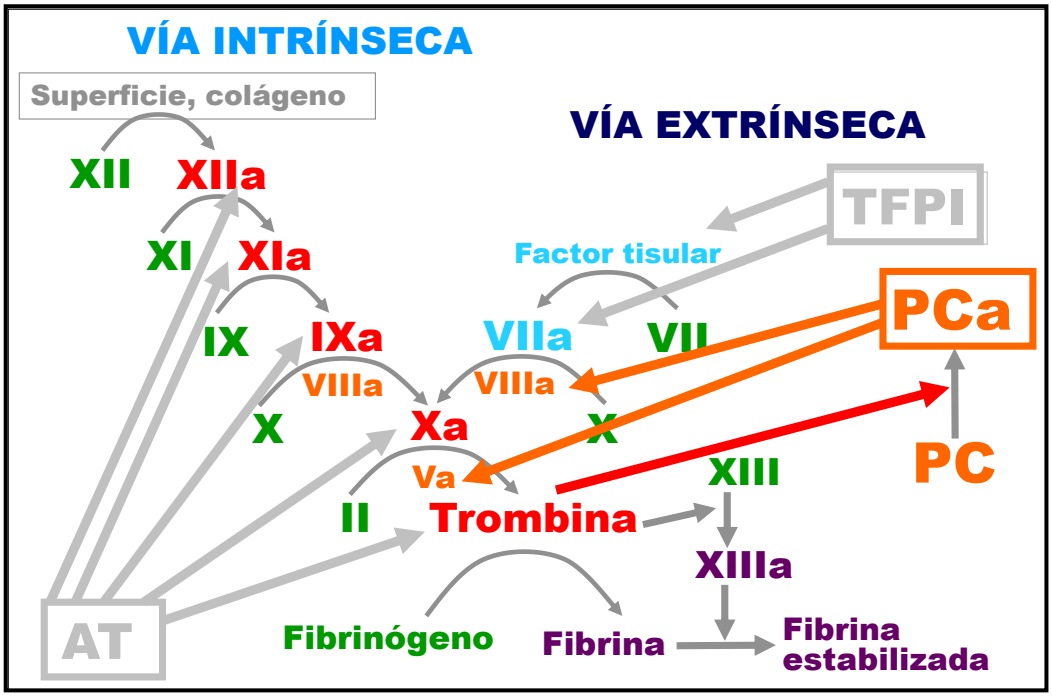

Figura 5. Inhibidores de la coagulación.
(PC: proteína C, PCa: proteína C activada,
TFPI: inhibidor de la vía del factor tisular, AT: antitrombina)

Además, existen otras proteínas que se unen a los fosfolípidos y que parecen ser capaces de regular la coagulación. Un ejemplo es la anexina V, que se une a superficies procoagulantes y las neutraliza, eliminando el soporte natural donde tienen lugar las reacciones de coagulación. Sin embargo, se desconoce la importancia fisiológica de su actividad anticoagulante.

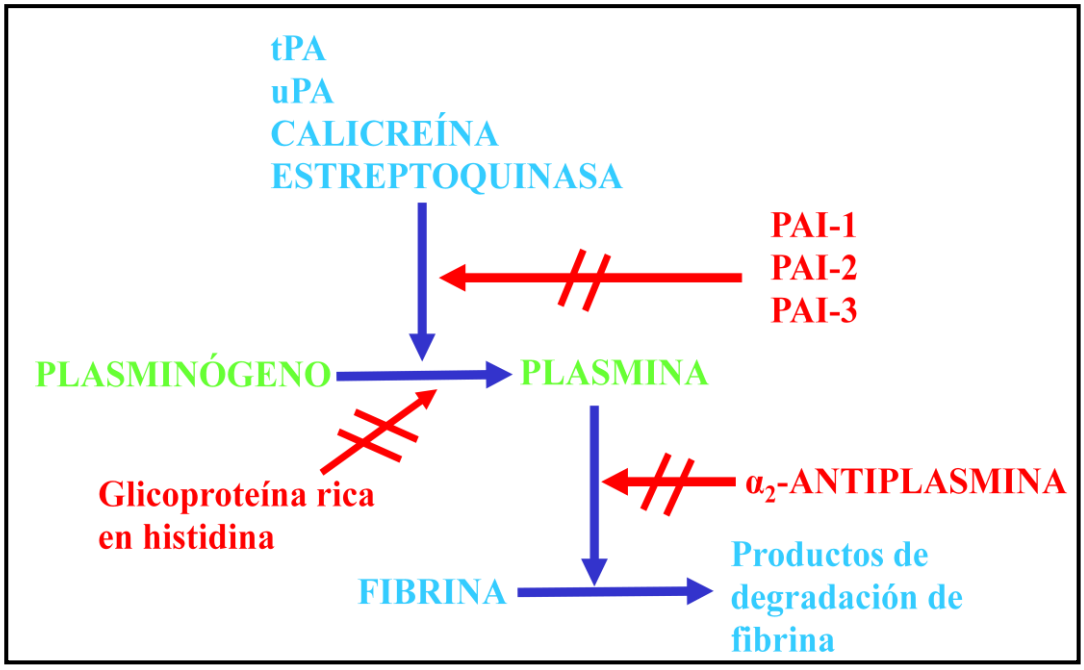

Figura 6. Sistema fibrinolítico.

Por otra parte, una vez el tapón hemostático ha realizado su función de detener o impedir la hemorragia, debe ser eliminado para restablecer el flujo normal de la sangre a través del vaso. De ello se encarga el llamado sistema fibrinolítico, cuyo principal componente es la plasmina (Figura 6). Ésta circula en la sangre en forma de proenzima inactivo o plasminógeno, que debe ser activado por sus activadores: tPA, uPA, la calicreína plasmática y la estreptoquinasa, este último activador se utiliza como fármaco, no se produce de forma endógena. Todos ellos son capaces de transformar al plasminógeno en el enzima activo o plasmina. El tPA, proveniente de la célula endotelial y es el principal activador del plasminógeno. Una vez activada, la plasmina degrada al coágulo de fibrina en pequeños fragmentos que son solubles en plasma, denominados productos de degradación de fibrina. A su vez, para controlar que esta disolución del coágulo no se realice antes de lo deseado, existen una serie de inhibidores que regulan todas las etapas anteriores. Así, los inhibidores de los activadores del plasminógeno, PAI-1, 2 y 3, inhiben a los activadores del plasminógeno, reduciendo la formación de plasmina. Por otra parte, la alfa-2-antiplasmina inhibe directamente a la plasmina. Finalmente, la glicoproteína rica en histidina inhibe la activación del plasminógeno de manera indirecta, al impedir la unión del plasminógeno a la fibrina.

1.3. Sistema de la proteína C

El sistema de la PC es un mecanismo natural de anticoagulación que regula la formación de trombina mediante la inactivación proteolítica de los cofactores de la coagulación FVa y FVIIIa (Esmon CT, 1987a & 1992 & 2003b; Esmon CT, 1987b). La PC es un zimógeno inactivo vitamina K-dependiente, precursor de la serinproteasa PC activada (APC). Su activación tiene lugar por acción de la trombina. Sin embargo, esta activación es muy lenta en la luz del torrente sanguíneo. Para la activación de la PC *in vivo*, la trombina debe unirse a un receptor específico situado en la superficie de la célula endotelial, la trombomodulina (TM) (Figura 7A), y este complejo activa eficientemente a la PC sobre la superficie de la célula endotelial. Además, la unión de la PC a otro receptor endotelial, el receptor endotelial de la PC (EPCR), acelera aún más la activación de la PC (Figura 7B). Una vez generada, la APC se disocia del EPCR y se une a su cofactor, la PS, una segunda proteína vitamina K-dependiente no enzimática, presente en la circulación y en las plaquetas. El complejo APC-PS inhibe, por degradación proteolítica, a los cofactores de la coagulación FVa y FVIIIa, reduciendo drásticamente la formación de trombina (Figura 7C). La PS circula en plasma en forma libre o acomplejada con un componente regulador del sistema del complemento (C4b-BP). Solo la forma libre es capaz de actuar como cofactor de la función anticoagulante de la APC. A su vez, la actividad anticoagulante de la APC está regulada por varios inhibidores, entre los que destacan la α_1-antitripsina (α_1-AT), el inhibidor de la PC (PCI) y la α_2-macroglobulina. Por otra parte, la trombina es fácilmente inactivada por la AT, quedando el complejo trombina-AT rápidamente disociado de la TM.

Además, una vez activada, la APC puede quedar unida a su receptor, el EPCR, y expresar de esta manera sus funciones citoprotectoras (Figura 7C). Así, el complejo APC-EPCR es capaz de activar a otro receptor endotelial, el receptor activado por proteasas tipo 1 (PAR-1), iniciando de esta manera una serie de señalizaciones intracelulares que desembocan en acciones antiinflamatorias, antiapoptóticas y neuroprotectoras (España F, 2005; Mosnier LO, 2007b; Jackson C, 2008).

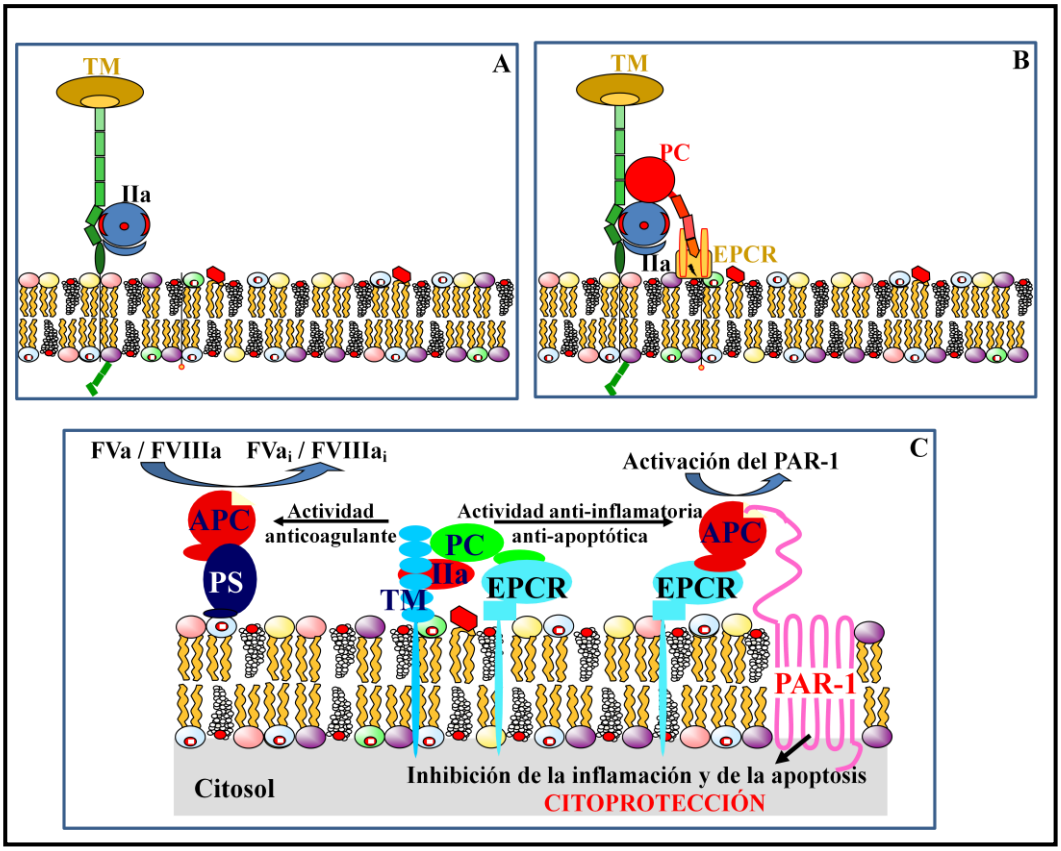

Figura 7. Activación y funciones de la Proteína C. La activación de la PC tiene lugar en la superficie de la célula endotelial, tras la formación de un complejo cuaternario de la trombina (IIa)-TM (A), PC y EPCR (B). Una vez activada, la APC puede disociarse de su receptor, unirse a su cofactor, la PS, y ejercer su función anticoagulante (C, izquierda). Si la APC queda unida a su receptor, EPCR, este complejo puede activar al receptor activado por PAR-1 y ejercer sus funciones citoprotectoras (C, derecha).

1.3.1. El receptor endotelial de la proteína C

El receptor endotelial de la PC (EPCR) (Esmon CT, 1999b; Esmon CT, 2000b) es una proteína integral de membrana de tipo I, que consta de 238 aminoácidos. Tras la eliminación de 17 residuos, se origina la proteína madura, formada por 221 aminoácidos. La proteína contiene una secuencia señal de 15 aminoácidos en el extremo N-terminal, un dominio extracelular con 4 sitios potenciales de N-glicosilación y 3 residuos de cisteína, una región transmembrana de 25 aminoácidos en el extremo C-terminal, y una cola citoplasmática corta compuesta únicamente por 3 aminoácidos, lo que prácticamente impide su capacidad de señalización. Estructuralmente, es una proteína homóloga de la

superfamilia de proteínas del complejo mayor de histocompatibilidad (MHC) tipo I/CD1, lo que sugiere un posible papel modulador de la inflamación y la inmunidad.

Se localiza principalmente en la membrana de la célula endotelial de grandes vasos y, además de la forma unida a la membrana, el EPCR es escindido del endotelio mediante la acción de metaloproteasas, activadas por mediadores inflamatorios (IL-1β), trombina, peróxido de hidrógeno o acetato de miristato forbol. Esta forma soluble de EPCR (sEPCR) carece del dominio transmembrana y la corta cola citoplasmática, está presente en el plasma y es capaz de unir PC y APC con la misma afinidad. La unión de la PC al sEPCR bloquea su activación al bloquear la interacción fosfolipídica y el sitio activo necesario para ello, mientras que la unión de la APC al sEPCR inhibe su función anticoagulante. Además, el sEPCR se une a neutrófilos, en un proceso donde están implicados la proteinasa 3 y el complejo CD11b/CD18 o Mac-1, sugiriendo la existencia de un vínculo entre la vía de activación de la PC y las funciones de los neutrófilos (Kurosawa S, 2000), que el sEPCR limitaría. Los niveles plasmáticos de sEPCR en la población general presentan una distribución bimodal (Stearns-Kurosawa DJ, 2003). Entre el 15-20% de la población tiene unos valores plasmáticos de sEPCR que varían entre 200 y 800 ng/mL, y el resto tiene valores por debajo de los 180 ng/mL. Determinadas patologías o situaciones patológicas aumentan los niveles de sEPCR, tales como lupus eritomatoso o sepsis (Kurosawa S, 1998).

Qu y colaboradores (Qu D, 2007) demostraron que la metaloproteasa ADAM17, inicialmente identificada como TACE (TNFα *converting enzyme*), regulaba el corte proteolítico del EPCR anclado a la membrana, localizando además la región para este corte entre los aminoácidos 192 y 200. Además, ADAM17 promueve el corte proteolítico de EPCR de la membrana y la liberación a la circulación de múltiples proteínas, destacando moléculas pro-inflamatorias y de adhesión (TNFα, V-CAM-1, L-Selectina, la proteína precursora β-amiloide, el receptor tipo II de IL-1, etc) (Qu D, 2007; Peiretti F, 2009). Posteriormente, se ha demostrado que existe otro mecanismo implicado en la generación de sEPCR, mediado sitios de corte y empalme de mRNA o *splicing* alternativos, y responsable de generar una isoforma truncada de sEPCR (Saposnik B, 2008; Molina E, 2008).

La expresión de EPCR es inhibida por lipopolisacáridos, IL-1β, TNFα y trombina. Y por otra parte, el EPCR es capaz de traslocarse al núcleo, ejerciendo acciones reguladoras de la respuesta inflamatoria, ya que regula la expresión génica de las

proteínas implicadas. En esta traslocación, el EPCR puede llevar unido APC (Esmon CT, 2003a & 2008).

Además de la función anticoagulante dependiente de PC, el EPCR tiene una acción anticoagulante independiente. El EPCR puede unirse al FVII/FVIIa, inhibiendo así la activación del FX (Preston RJ, 2006; Ghosh S, 2007; López-Sagaseta J, 2007). Por otra parte, la unión del FVIIa al EPCR produce un cambio conformacional en el factor de la coagulación que facilita su endocitosis y eliminación de la circulación (Ghosh S, 2007). Tanto la PC como el FVIIa tienen similar afinidad de unión por el EPCR, y ambos se unen por la misma región del dominio Gla, de manera que compiten por su unión al EPCR (Preston RJ, 2006; Ghosh S, 2007; López-Sagaseta J, 2007). Sin embargo, la concentración fisiológica de la PC en plasma es de 70 nM mientras que la del FVII es de 10 nM, por lo que la generación de APC se ve mínimamente afectada por la presencia del FVII. Aunque, en ciertos tratamientos con FVIIa recombinante, las concentraciones farmacológicas de FVIIa alcanzadas podrían inhibir parcialmente la activación de la PC dependiente del EPCR y la señalización celular mediada por la APC, aumentando el efecto procoagulante del FVIIa recombinante (Ghosh S, 2007; López-Sagaseta J, 2007). Además, Molina y colaboradores, demostraron que la isoforma truncada de sEPCR que habían identificado, también es capaz de unirse al FVIIa, inhibiendo así la activación del FX y contrarrestando el efecto proinflamatorio del sEPCR vía unión PC/APC (Molina E, 2008).

1.3.2. Estructura del gen que codifica para el EPCR

El gen del EPCR, también conocido como *PROCR*, está localizado en el cromosoma 20, se expande a lo largo de 8 Kb y está constituido por 4 exones (Fukudome K & Esmon CT, 1994; Esmon CT & Fukudome K, 1995b; Simmonds RE & Lane DA, 1999; Hayashi T, 1999). El sitio de inicio de la transcripción está situado 79 pb *upstream* del codón de inicio de la traducción (Met) y 84 pb *downstream* de una secuencia reguladora tipo caja TATA. A su vez, existen potenciales dianas de unión de los factores de transcripción AP1, SP1 y AP2 en la región promotora del gen, dos elementos reguladores CAAT (posiciones –436 y –243 del inicio de la transcripción) y un sitio de reconocimiento para *enhancer binding protein* (posición –462 del inicio de la

transcripción). El exón 1 (138 pb; aminoácidos 1 – 24) codifica para la región 5' no traducida, el péptido señal y 7 aminoácidos adicionales; el intrón I tiene una longitud de 2477 pb y contiene una secuencia repetitiva Alu (nt 1230 – 1590); el exón 2 (252 pb; aminoácidos 24 – 108) codifica para parte de la región extracelular del receptor; el intrón II tiene una longitud de 1217 pb y contiene un elemento repetitivo Alu (nt 3417 – 3747); el exón 3 (279 pb; aminoácidos 108 – 201) codifica para el resto de la región extracelular del EPCR; el intrón III tiene una longitud de 251 pb; y el exón 4 (659 pb; aminoácidos 201 – 238) codifica para 10 residuos adicionales de la región extracelular, el dominio transmembrana, la cola citoplasmática corta y la mayor parte de la región 3' no traducida. Se han identificado 2 sitios alternativos de poliadenilación en la región 3' del gen (Simmonds RE & Lane DA, 1999).

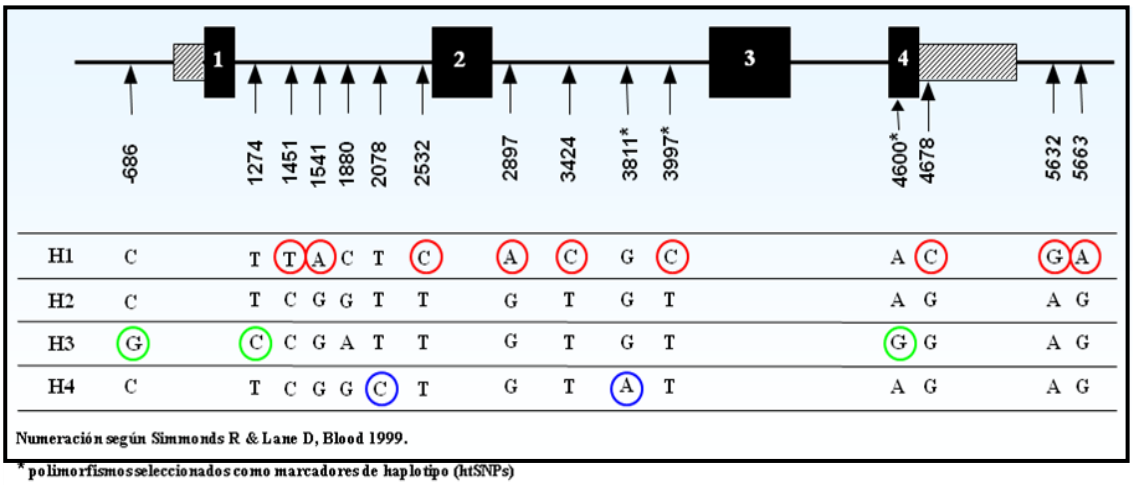

Figura 8. Haplotipos del gen de EPCR (*PROCR*)

1.3.3. Haplotipos del gen *PROCR*

Se ha descrito la existencia de cuatro haplotipos en el gen *PROCR* (Figura 8), con diferentes polimorfismos marcadores de haplotipo para tres de los cuatro haplotipos descritos. El haplotipo 1 (H1), identificado mediante el alelo mutado para el polimorfismo 4678G/C (rs9574) localizado en la región 3' no traducible del EPCR, que está asociado con elevados niveles de APC circulante y una disminución del riesgo de tromboembolismo venoso (TEV) (Medina P, 2004 & 2005, Pecheniuk 2008). El mecanismo por el cual aumenta los niveles de APC y reduce el riesgo trombótico se

desconoce, aunque se supone que en presencia del alelo mutado se estabiliza el mRNA, sintetizándose una mayor cantidad de EPCR en la membrana de la célula endotelial, que produce una mayor activación de la PC y mayores niveles de APC circulantes. Aunque otros autores no han encontrado dicho efecto sobre el riesgo trombótico (Saposnik 2004, Uitte 2004), pero dichas discrepancias podrían deberse a diferentes poblaciones y diseños de estudio, algunos de ellos con escaso número de pacientes.

El haplotipo 1 (H1) contiene más de 10 SNPs específicos de haplotipos y se desconoce cuál es el responsable del efecto sobre el EPCR.

El haplotipo 2 (H2) se corresponde con la secuencia original que contiene los alelos más frecuentes.

El haplotipo 3 (H3), identificado mediante el alelo mutado para el polimorfismo g.4600A>G (Ser219Gly; rs867186), localizado en el exón 4 del gen *PROCR* y genera el cambio de aminoácido de Ser a Gly en posición 219 en el EPCR. Está asociado con un aumento de los niveles de sEPCR circulante, posiblemente debido a que en presencia del alelo 4600G se produce un cambio conformacional que aumenta la sensibilidad de ADAM17 por el EPCR en la superficie de la célula endotelial, aumentando el corte proteolítico sobre el EPCR (Qu D, 2007, Saposnik B, 2008). Puesto que el sEPCR puede unir con la misma afinidad FVII/FVIIa y PC/APC, (Preston RJ, 2006; Ghosh S, 2007; López-Sagaseta J, 2007), se podría pensar que H3, mediante su asociación con elevados niveles de sEPCR, podría estar asociado con un mayor riesgo de TEV. Los resultados descritos en diferentes estudios son contradictorios. En sujetos franceses, se ha descrito una asociación entre la presencia de H3 y un aumento del riesgo de TEV en hombres (Saposnik B, 2004). Sin embargo, otros trabajos no han sido capaces de mostrar una asociación entre H3 y el riesgo de TEV, tanto en sujetos españoles (Medina P, 2004 & 2005), holandeses (Uitte de Willige S, 2004) o estadounidenses (Pecheniuk NM, 2008). El efecto trombótico de H3 podría observarse potenciado en presencia de otras trombofilias o situaciones protrombóticas, como se ha demostrado en caso de coexistir con el alelo 20210A de la protrombina (Navarro S, 2008) o en caso de TEV durante la gestación o el puerperio (Galanaud JP, 2010). Por el contrario, Ireland y colaboradores (Ireland, 2005) demostraron en un estudio caso-control en pacientes con infarto de miocardio, que los individuos portadores de H3 tenían un menor riesgo de infarto de miocardio, excluyendo individuos con diabetes y síndrome metabólico.

El haplotipo 4 (H4), identificado mediante el alelo mutado para el polimorfismo 3811G/A localizado en el intrón II del gen *PROCR*, está ligeramente asociado con aumento del riesgo trombótico (Uitte de Willige S, 2004).

1.3.4. El receptor endotelial de la trombina: la trombomodulina

La trombomodulina (TM) es el receptor situado en la superficie de la célula endotelial específico de la trombina. Cuando la trombina se une a la TM pierde sus propiedades procoagulantes y se convierte en un potente anticoagulante, al activar eficientemente a la PC. Ello es así porque dicha unión se realiza por el mismo sitio por el que la trombina ejerce sus funciones procoagulante. La TM pertenece al grupo de glicoproteínas de membrana tipo I, y se sintetiza como un precursor de 575 aminoácidos incluyendo un péptido señal de 18 residuos. Tras la eliminación del péptido señal se genera la proteína madura, con una masa molecular de 74 kD y compuesta por 557 aminoácidos. Estructuralmente es una proteína similar al receptor LDL, está formada por un dominio globular N-terminal tipo lectina (aminoácidos 1-154), seguido de una región hidrofóbica (aminoácidos 155-222), una región homóloga al factor de crecimiento epidérmico (EGF) (aminoácidos 223-462), una región rica en serina-treonina (aminoácidos 463-497) que contiene sitios de unión para modificaciones post-transcripcionales para N- y O-glicosilaciones, y en especial la adición de condroín sulfato, un dominio transmembrana (aminoácidos 498-521) y una corta cola citoplasmática carboxi-terminal (aminoácidos 522-557) (Shirai T, 1988). La región rica en serina-treonina parece actuar como región espaciadora permitiendo la correcta localización de la trombina sobre la membrana para la unión de la TM, facilitando su actividad anticoagulante (Hancock W, 1992).

La TM se localiza mayoritariamente en la membrana del endotelio vascular, pero también está presente en múltiples tejidos y células, incluyendo astrocitos, queratinocitos, células mesoteliales, neutrófilos, monocitos y plaquetas, aunque en pequeñas cantidades. En el endotelio vascular, la TM se localiza fundamentalmente en la microvasculatura, inhibiendo la actividad procoagulante de la trombina.

Además de la TM anclada a la membrana endotelial, se han descrito formas solubles de TM en plasma y orina. Esta TM soluble (sTM) es la forma libre en plasma

que carece de parte de la zona rica en serinas-treoninas, y también carece del dominio transmembrana y la cola citoplasmática. La concentración de la sTM aumenta en determinados estados y patologías asociadas a disfunción endotelial, enfermedades cardiovasculares, diabetes, daño isquémico, inflamación, hiperhomocisteinemia, fallos renales, daño pulmonar, sepsis, vasculitis, enfermedades del colágeno, malaria...Por ello, los niveles de sTM se pueden utilizar como marcador de daño endotelial. Existen estudios que atribuyen a la sTM propiedades protectoras del sistema vascular (Van der Wouwer, 2004).

1.3.5. Estructura del gen que codifica para la TM

El gen de la TM (*THBD*) se localiza en posición 20p11.2 y carece de secuencias intrónicas. Mutaciones en el gen *THBD* que alteren la expresión o activación de la PC pueden predisponer a eventos trombóticos, pero dichas mutaciones son infrecuentes en la población general, seguramente debido a que la pérdida de las funciones de la TM debe ser letal a nivel embrionario. Existe un polimorfismo descrito en la región codificante del gen *THBD* (c.1418C>T) (rs1042579) que origina el cambio del aminoácido alanina por valina en la posición 455, el cual podría tener un papel importante en la función de la TM ya que el aminoácido se encuentra en la región de la proteína que se une a la trombina y activa la PC.

1.3.6. Polimorfismo del gen *THBD*

Existen estudios que han intentado demostrar al asociación del polimorfismo c.1418C>T del gen *THBD* con la trombosis venosa o arterial, pero los resultados son controvertidos. Heit y colaboradores (Heit JA, 2005) no encuentran asociación entre el polimorfismo y el riesgo de TEV. El estudio LITE (Aleksic N, 2003) solo consigue demostrar un menor riesgo de TEV en la población negra portadora del alelo 1418T. En cambio, en un estudio realizado en población japonesa (Sugiyama S, 2007), observaban mayor riesgo de trombosis en presencia del alelo 1418T. También existen publicaciones contradictorias en el caso de trombosis arteriales en las que muestran que el alelo 1418T

tiene un efecto protector (Norlund L, 1997) o bien que dicho alelo aumenta el riesgo de padecer estos eventos (Konstantoulas CJ, 2004). Una posible explicación a estas discrepancias podría ser la diferencia en el diseño de los experimentos y las poblaciones empleadas. Los estudios nombrados tenían bajo número de pacientes (entre 18 y 302 según el estudio) y además, la población escogida para el estudio era de edad avanzada.

1.4. Enfermedad de Behçet

La enfermedad de Behçet, descrita en 1937 por el Dr. Hulusi Behçet, se define como un desorden inflamatorio crónico de origen desconocido que afecta a los vasos sanguíneos, produciendo una vasculitis sistémica recurrente en venas, arterias y capilares sanguíneos, y se caracteriza por la presencia de aftas orales y genitales, uveítis y lesiones cutáneas. Aparece en individuos de entre 30 y 40 años (Benamour S, 1999), expuestos a algún agente externo medioambiental, probablemente una bacteria, y genéticamente predispuestos, siendo infrecuente en niños e individuos de más de 50 años. Se ha descrito que los pacientes en quienes la enfermedad se manifiesta a edades más tempranas desarrollan cuadros más graves (Pipitone N, 2004). Afecta a ambos géneros, aunque en hombres jóvenes aparecen los síntomas más severos, con afecciones oculares, vasculares y neurológicas, de mayor mortalidad (Yazici H, 1984).

Se diagnostica de acuerdo al criterio definido por el Grupo Internacional de Estudio de la Enfermedad de Behçet (*Criteria for diagnosis of Behçet´s disease. Internacional Study Group for Behçet´s Disease*, 1990), según la presencia de ulceraciones orales y genitales recurrentes, inflamación ocular y lesiones cutáneas. Otras manifestaciones que pueden desarrollar estos pacientes son: inflamación en articulaciones, artralgias o artritis, inflamación intestinal, inflamación del sistema nervioso tanto a nivel central como periférico, afectación audiovestibular, pulmonar, hepática o cardíaca, tromboflebitis subcutáneas, trombosis venosa o arterial y/o aneurismas (Yazici H, 1998).

Aunque tiene una amplia distribución geográfica, aparece con más frecuencia desde el este de Asia hasta los países del este del mar Mediterráneo, siendo infrecuente en el centro y norte de Europa. En España la frecuencia se cifra entre 5,6 y 6,4 casos por 100.000 habitantes (Graña J, 2001; Registro de 1978 a 1990, La Coruña; González-Gay MA, 2000; Registro de 1988 a 1997, Lugo).

1.4.1. Coagulación, inflamación y enfermedad de Behçet

Los pacientes con Behçet presentan un estado procoagulante (Kiraz S, 2002) asociado a la inflamación y el daño en el endotelio vascular que acompaña a la enfermedad, y que produce además una elevada generación de trombina y una menor fibrinólisis. Además, los pacientes con Behçet se caracterizan por la presencia de niveles reducidos de tPA (Orem A, 1995) y TM (Espinosa G, 2002; Yurdakul S, 2005; Ricart JM, 2008).

Entre el 25 y 30% de los pacientes con Behçet sufren trombosis, siendo el tromboembolismo venoso más frecuente que el arterial. Sin embargo, el mecanismo que explica la formación del trombo en la enfermedad de Behçet todavía es desconocido, debiendo contribuir otros factores ambientales y hereditarios, puesto que aunque las alteraciones producto de la inflamación y daño endotelial son un factor de riesgo trombótico, no son suficientes para explicarlo, ya que otras enfermedades ligadas a vasculitis no causan trombosis, y no todos los pacientes con Behçet la sufren (Lie J, 1992; Espinosa G, 2002).

La etiología de la trombosis en la enfermedad de Behçet resulta desconocida, pero resultados previos descritos por nuestro grupo (España F, 2001) mostraron que el sistema anticoagulante de la PC influye en el desarrollo del evento trombótico en pacientes con dicha enfermedad, constituyendo un factor de riesgo trombótico. Por consiguiente puede ser que esta mayor predisposición trombótica tenga un componente genético relacionado con los haplotipos en el gen *PROCR*, dado que observamos un nivel reducido de APC en los pacientes con enfermedad de Behçet.

2. OBJETIVOS

Los objetivos planteados son:

- Conocer la asociación del polimorfismo c.1418C>T del gen *THBD* en un estudio caso-control en pacientes que habían sufrido un TEV y comparar sus niveles plasmáticos de TM y APC. Con el fin de profundizar en la funcionalidad del polimorfismo, se aislaron células endoteliales de vena umbilical humana (HUVEC) para analizar la relación entre dicho polimorfismo y la cantidad de mRNA del gen *THBD* y la expresión proteica de la TM.
- Verificar el efecto de H1 y H3 del gen *PROCR* en el riesgo del TEV en un estudio caso-control en pacientes que habían sufrido un TEV, conocer su repercusión sobre la expresión del gen *PROCR* en HUVEC e identificar el SNP funcional de H1 que explicaría su efecto.
- Dado que se había observado un nivel reducido de APC en los pacientes con enfermedad de Behçet, y puesto que estos niveles pueden estar influenciados por los haplotipos en el gen *PROCR*, analizamos la asociación entre H1 y H3, los niveles plasmáticos de APC y sTM, y el riesgo de TEV en un grupo de pacientes con la enfermedad de Behçet.

3. MATERIAL Y MÉTODOS

El diseño de los diferentes estudios, los métodos empleados y los análisis estadísticos se describen detalladamente en los artículos.

Los pacientes reclutados para el estudio de la TM y el EPCR habían sufrido, al menos, una trombosis venosa profunda. Las trombosis se catalogaron como embolismos pulmonares, retrombosis, trombosis familiares y trombosis espontáneas (es decir, tras excluir factores de riesgo circunstanciales como embarazo, trauma, cirugía, inmovilización o anticonceptivos orales). Los controles sanos no presentaban históricos de trombosis y se obtuvieron de donantes de los hospitales de Murcia, Salamanca y Valencia. Todos ellos presentaban una edad y sexo similar a los pacientes. La prevalencia de los polimorfismos clásico de trombosis (mutaciones FV Leiden y protrombina 20210A) fue similar a la descrita en otras series.

Las HUVEC se obtuvieron de cordones umbilicales de caucásicos recién nacidos. Los cordones fueron digeridos con colagenasa para poder liberar las células endoteliales de la vena umbilical y así llevar a cabo su cultivo en frascos estériles. Los polimorfismos de los genes *THBD* y *PROCR* fueron genotipados por secuenciación directa y la cuantificación del mRNA de los diferentes transcritos se estudió por PCR cuantitativa a tiempo real. Se recogieron los medios de cultivo para el estudio proteico de las fracciones solubles de TM y EPCR, y posteriormente, se lisaron las células para obtener los lisados celulares y cuantificar los niveles proteicos intracelulares. El experimento de activación de la PC sobre las HUVEC se llevó a cabo tras incubación de las células con PC y trombina de origen bovino, en presencia y ausencia de un anticuerpo monoclonal murino anti-EPCR, seguido de la medición de la actividad amidolítica de la APC generada (España F, 1996 & 2001).

Los 87 pacientes con la enfermedad de Behçet fueron diagnosticados en el Hospital Universitario y Politécnico la Fe y el Hospital General de Valencia, y cumplían los criterios del Grupo Internacional de Estudio de la enfermedad de Behçet (*Criteria for diagnosis of Behçet´s disease. Internacional Study Group for Behçet´s Disease*, 1990). Tres de los pacientes presentaban afectación ocular severa en el momento de la recogida de muestras, lo que indica que estaban en una fase aguda de la enfermedad y fueron excluidos del estudio. El resto de pacientes estaban en una fase latente de la enfermedad o

con patología mínima (aftas bucales o artralgias). Diecinueve de los pacientes habían sufrido un TEV. El grupo control estaba formado por 260 individuos de similar distribución de edad y sexo al de los pacientes, que fueron recogidos de forma paralela procedentes de controles rutinarios en nuestro centro, todos ellos estaban aparentemente sanos y no presentaban historia personal ni familiar de trombosis.

4. RESULTADOS

4.1. Estudio del polimorfismo c.1418C<T del gen *THBD*

4.1.1. Relación entre el polimorfismo c.1418C>T del gen THBD y el riesgo de TEV

Se genotipó el polimorfismo c.1418C>T del gen *THBD* en 1173 pacientes y 1262 controles sanos. Entre los pacientes sanos se encontró una frecuencia alélica similar a la descrita en otros estudios (van der Velden PA, 1991; Heit JA, 2005; Konstanoulas CJ, 2004), pero era más baja que la descrita por otros (Ohlin AK, 1995; Norlund L, 1997), posiblemente debido a las diferentes localizaciones geográficas de las poblaciones y al tamaño muestral de alguno de los estudios. Para descartar la posible influencia del origen geográfico de los controles, se analizó la frecuencia alélica en las tres poblaciones (Valencia, Murcia y Salamanca) y los resultados fueron similares entre ellos. Se procedió de forma similar con los pacientes que mostraron una menor frecuencia alélica que los controles. En el análisis univariante se mostró que la presencia del alelo 1418T del gen *THBD* o el genotipo TT, reducía significativamente el riesgo de TEV (OR: 0,74; IC 95%: 0,63-0,87 y OR: 0,56; IC 95%: 0,32-0,96, respectivamente). Tras ajustar por edad, sexo y presencia de defectos trombofílicos (factor V Leiden, protrombina 20210A, déficit de PC, PS o AT), el análisis multivariante no mostraba cambios significativos en las ORs. El análisis ajustado en pacientes con retrombosis (OR para genotipo TT en retrombosis: 0,41; IC 95%: 0,14-1,20, sin retrombosis: 0,56; IC95%: 0,30-1,05) o en pacientes con presencia de defectos trombofílicos (OR para genotipo TT con factores de riesgo: 0,60; IC 95%: 0,26-1,24, sin factores de riesgo: 0,48; IC95%: 0,25-0,92) mostraba ORs sin variaciones significativas.

Se agruparon los pacientes y los controles en dos grupos según tuvieran más o menos de 45 años, viéndose que el efecto protector del alelo 1418T frente a la trombosis era más pronunciado en los más jóvenes (OR ajustado para genotipos CT y TT: 0,55; IC 95%: 0,43-0,71), mientras que el efecto protector del alelo T desaparecía en el grupo de mayores de 45 años (OR ajustado para genotipos CT y TT: 1,06; IC 95%: 0,81-1,39).

4.1.2. Relación entre los niveles de sTM en plasma y el riesgo de TEV

Con el fin de conocer si los niveles de sTM en plasma podrían estar asociados con riesgo de TEV, comparamos los niveles de sTM en 414 pacientes que habían sufrido un evento trombótico y 451 controles sanos. Fueron excluidos aquellos pacientes que estaban bajo tratamiento con anticoagulantes orales. Se encontró que los niveles de sTM eran significativamente más altos en pacientes que en controles (P=0,005). A continuación, para conocer si dichos niveles podrían explicarse en relación con el alelo 1418T del gen *THBD*, estratificamos los niveles de sTM en función del genotipo para el polimorfismo c.1418C>T y la edad, según tuvieran menos o más de 45 años, y en ambos grupos de pacientes y controles. Observamos que los niveles de sTM eran significativamente menores a medida que el número de alelos T1418 aumentaba (P<0,001).

En base a los anteriores resultados, parecía que los niveles de sTM están asociados con el polimorfismo c.1418C>T del gen *THBD*. Por tanto, realizamos un análisis multivariante que incluía las variantes siguientes: edad, sexo, presencia de factores trombofílicos, niveles de sTM <3,86 ng/mL y presencia del alelo 1418T. El valor de corte para los niveles de la sTM correspondía al percentil 50 de la distribución de los valores de sTM en los controles. La presencia del alelo 1418T (incluye tanto portadores de un alelo como de dos, genotipo CT o TT) presentaba un OR de 0,75 (0,62-0,90) en ausencia de los niveles de sTM como factor, y un OR de 0,61 (0,45-0,84) incluyendo los niveles de sTM en el modelo. Por otro lado, el OR para la sTM<3,86 ng/mL era de 0,65 (0,49-0,86) sin la presencia del alelo 1418T y 0,61 (0,45-0,84) incluyendo el alelo 1418T en el modelo. Estos resultados nos sugieren que tanto la presencia del alelo 1418T como niveles disminuidos de sTM protegen frente al TEV de forma independiente.

4.1.3. Niveles de Proteína C activada en relación al polimorfismo del gen THBD

Una posible explicación del mecanismo por el cual los portadores del genotipo 1418TT para el gen *THBD* presentan menor riesgo de TEV podría ser el aumento de los niveles de APC en el plasma. Por tanto, se procedió a determinar los niveles de APC en el plasma de 451 controles sanos y 414 pacientes que habían sufrido un evento trombótico, excluyendo aquellos que estaban bajo tratamiento con anticoagulantes orales. Tal como había sido descrito en anteriores estudios por nuestro grupo (España F, 2001), los niveles de APC eran menores en pacientes que en controles. Al distribuir los niveles de APC

según los genotipos, encontramos que tanto en pacientes como en controles existía una tendencia a presentar niveles de APC mayores a medida que los individuos tenían mayor número de alelos 1418T, pero dado que el número de pacientes con el genotipo TT era muy bajo, las diferencias observadas no fueron significativas.

Se observó una correlación inversa entre los niveles de APC y los niveles de sTM en los controles (r=-0,161; P=0,001).

4.1.4. Estudio del polimorfismo del gen THBD en HUVEC

Proseguimos con la obtención de HUVEC con el objetivo de poder estudiar la funcionalidad del polimorfismo c.1418C>T del gen *THBD*. Se recogieron 100 cordones umbilicales de los que se aislaron las HUVEC que fueron genotipadas; éstas presentaban los siguientes genotipos: 3 presentaban el genotipo 1418TT, 26 presentaban el genotipo 1418CT y 71 presentaban el genotipo 1418CC.

Se cuantificó la sTM en el medio de cultivo de las HUVEC aisladas a partir de los cordones umbilicales mediante ELISA. Los niveles de sTM en el medio se podrían equiparar a los niveles presentes en plasma. En los lisados celulares se cuantificó la TM unida a la membrana mediante ELISA y *Western blot*. Esta TM unida a la membrana sería la equivalente a la TM presente en el endotelio vascular *in vivo*. A medida que el número de alelos 1418T aumentaba, los niveles de TM unida a membrana aumentaban (P<0,001) y los niveles de sTM en el medio disminuían (P<0,001). Los resultados del *Western blot* confirmaron los resultados obtenidos por ELISA, ya que se observaba mayor concentración de TM en los lisados celulares cuando aumentaba el número de alelos 1418T (P<0,001).

Además, se determinaron los niveles de mRNA del gen *THBD* en 95 HUVEC. Los niveles de mRNA del gen *THBD* no presentaron diferencias significativas al comparar los distintos genotipos: 7,5 (4,1-8,9) para los 67 HUVEC con el genotipo 1418CC, 6,1 (3,6-6,6) para los 26 HUVEC con el genotipo 1418CT y 6,8 y 3,0 para los dos HUVEC con genotipo 1418TT (P=0,496).

Con el objetivo de estudiar la funcionalidad de la TM *in vivo*, cuantificamos la activación de la PC en las HUVEC. Para ello se utilizaron dos muestras de cada genotipo, y todas ellas fueron seleccionadas con el mismo genotipo para el gen *PROCR* (H1H2). Se observó que el ratio de activación de la PC aumentaba paralelamente con el aumento de

los alelos 1418T. Así, las células con el genotipo CC tenían solo un 60% de la activación que se observaba en las células TT. Para confirmar que el efecto que se observaba se debía solamente a la TM y no al EPCR, se repitió el análisis bloqueando el EPCR con un anticuerpo RCR-379, específico para EPCR. Como se podría prever, el bloqueo del EPCR disminuyó drásticamente la activación de la PC, aunque seguía existiendo una activación residual en la que se observaba una mayor activación de la PC conforme aumentaba la presencia del alelo 1418T.

4.2. Estudio de los haplotipos del gen *PROCR*

4.2.1. Relación entre los haplotipos del gen PROCR y el riesgo de TEV

Se genotiparon los H1 y H3 del gen *PROCR* en 702 pacientes que habían sufrido un evento trombótico y 518 controles sanos. Con el fin de estudiar la asociación entre los diferentes genotipos y el riesgo de TEV, se realizó un análisis de regresión logística. El análisis univariante confirmó la información aportada en estudios previos que indicaban que la presencia del genotipo H1H1 reduce significativamente el riesgo de TEV (Medina 2004, Medina 2007). En el análisis multivariante, tras realizar el ajuste por sexo, edad y presencia de defectos trombofílicos, no se observaron cambios significativos en las ORs (OR para H1H1: 0,66; IC 95%: 0,46-0,93 y tras ajustar, OR: 0,62; IC 95%: 0,43-0,89). También se observó que la presencia del genotipo H3H3 está asociada con un aumento del riesgo de TEV (OR: 12,07; IC 95%: 0,69-211,75).

4.2.2. Relación entre los haplotipos del gen PROCR y niveles de PC, APC y sEPCR

Se determinaron los niveles de PC, APC y sEPCR en el plasma de 462 pacientes que habían sufrido un evento trombótico, excluyendo aquellos en tratamiento con anticoagulantes orales, ya que la anticoagulación afecta a los niveles de PC y APC, y 510 controles sanos. Los niveles los APC circulantes eran inferiores en pacientes (1,03±0,37 ng/mL) que en controles (1,25±0,40 ng/mL; $P<0,001$), tal como demostraban investigaciones anteriores de nuestro grupo (España F, 2001). Tanto en pacientes como en controles, los niveles de APC aumentan con el número de alelos H1 ($P<0,001$), mientras que tienden a disminuir al aumentar el número de alelos H3.

Los niveles de sEPCR en plasma fueron similares en pacientes y controles. Cuando los niveles de sEPCR se distribuyeron en función del genotipo, los niveles de sEPCR aumentaron significativamente conforme aumentaba el número de alelos H3 ($P<0,001$),

lo cual confirma resultados anteriores (Medina P, 2004; Saposnik B, 2004; Uitte de Willige S, 2004; Medina P, 2007; Navarro S, 2008 & 2011; Yamagishi K, 2009; Reiner AP, 2008; Ireland H, 2005; Qu D, 2006). No se observaron variaciones de los niveles de sEPCR respecto al alelo H1.

Tal como había sido descrito anteriormente (Reiner AP, 2008; Tang W, 2010), tanto en pacientes como en controles, los niveles de PC antigénica eran significativamente mayores en los portadores del alelo H3 que en aquellos individuos no portadores de H3 (HxHx, HXH1, H1H1, siendo x=2 o 4). Estos datos indican que el aumento de los niveles de APC en los portadores de H1 no puede explicarse por un aumento en los niveles de PC antigénica en plasma.

4.2.3. Estudio de los haplotipos del gen PROCR en HUVEC

Con el fin de conocer la funcionalidad de H1 y H3 del gen *PROCR* y poder profundizar en los mecanismos asociados a los diferentes cambios genotípicos, utilizamos 111 cordones umbilicales humanos de los que aislamos las HUVEC y se cultivaron en frascos hasta un máximo de dos pases. Las HUVEC fueron genotipadas: 34 HUVEC fueron HxHx, siendo x los haplotipos 2 o 4, 36 fueron HxH1, 27 H1H1, 5 H1H3, 8 HxH3 y 1 H3H3.

Se cuantificaron los niveles de EPCR en el lisado celular que correspondería a los niveles de EPCR en la membrana endotelial, así como los niveles de EPCR en el medio de cultivo, que se podrían corresponder con los niveles de sEPCR en plasma. Los resultados mostraron que a mayor número de alelos H1, los niveles de EPCR en la membrana aumentaban (P<0,001), y los de sEPCR disminuían (P=0,024). La cantidad total de EPCR (membrana + soluble) aumentaba a medida que lo hacían los alelos H1 (P=0,004). Por el contrario, la presencia del alelo H3 se asociaba con niveles disminuidos de EPCR en membrana (P=0,004) y aumentados de sEPCR (P=0,080), como cabría esperar.

Paralelamente se midieron los niveles de mRNA del gen *PROCR*, tanto la fracción completa como el mRNA truncado, en 90 de los cultivos de HUVEC transcurrido un solo pase desde el aislamiento de las células. Se observó una tendencia no significativa de niveles ligeramente disminuidos de mRNA de longitud completa a medida que aumentaban los alelos H1 o H3. El mRNA truncado, tal como había sido descrito con

anterioridad (Saposnik B, 2008; Molina E, 2008), se pudo aislar y cuantificar, oscilando su concentración entre 0,3-0,5% en los cultivos de HUVEC no portadores de H3 y entre 1% y 2,3% en las células con el alelo H3. No obstante, este discreto aumento en los niveles de mRNA truncado no podría explicar los elevados niveles de sEPCR en los portadores del alelo H3.

A continuación, y para poder comprobar la funcionalidad del EPCR unido a membrana, se realizaron experimentos de activación de la PC sobre las HUVEC cultivadas para cada haplotipo. Como previamente habíamos demostrado que el polimorfismo c.1418C>T del gen *THBD* está relacionado con la activación de la PC, todas las HUVEC que fueron seleccionadas para este experimento eran homocigotas para el alelo más frecuente del polimorfismo del gen *THBD* (1418C). Los resultados mostraron que la activación de la PC era mayor a medida que aumentaba la presencia de los alelos H1 (P=0,007), y disminuía con la presencia del alelo H3 (P=0,029). Con el fin de demostrar que el cambio en la activación de la PC se debía al EPCR, se incubaron las HUVEC con un anticuerpo monoclonal específico para EPCR (RCR-379), y se observó un descenso drástico de la activación de la PC. Tras el experimento se recogieron las fracciones citosólicas y de membrana de las células, hallándose niveles de EPCR en membrana mayores para los portadores de H1 y menores en el caso de H3, tal como cabría esperar. Los niveles de EPCR en la fracción citosólica eran indetectables (<0,3 ng/mL).

4.2.4. Estudios funcionales in vitro del gen PROCR con H1

H1 se expande en una región mayor de la delimitada por el gen *PROCR*, pero dado que no han sido identificados elementos funcionales fuera de la región codificante del gen, limitamos el estudio de los SNPs funcionales a aquellos que codifican el gen. Para estudiar los SNPs específicos de H1 del intrón 1, 2 y de la región 3', generamos 3 construcciones con luciferasa donde clonamos el intrón 1, el intrón 2 o la región 3' no codificante de H1 y H2, siendo los clones de H2 utilizados como referencia. Tras transfectar las células endoteliales humanas (EC-RF24) por electroporación, se midió la actividad de la luciferasa. El intrón 1 de H1 mostró una reducción de un 45% en la actividad luciferasa comparado con el intrón 1 de H2 (P=0,029), el resto de regiones mostraron disminuciones no significativas respecto a las de referencia. Por tanto, los

resultados indicaron que el SNP funcional se encontraba en el intrón H1 de *PROCR*. A continuación, se generaron mediante mutagénesis dirigida, 4 construcciones donde habían sido clonados cada SNP de H1 de forma individual, utilizando H2 como referencia, y de nuevo se transfectaron las células EC-RF24. Solamente la construcción que contenía el alelo 2532C (rs2069948) mostraba una reducción de la actividad luciferasa de un 34,4% comparado con H2 (*P*=0,003). Al realizar una construcción que contenía los cuatro SNPs del intrón 1, se observó una disminución de la actividad luciferasa similar a la obtenida con el cambio del alelo 2532C (36,7%, *P*=0,006). Por lo tanto, se puede deducir que el SNP g.2532T>C parece ser el responsable de los efectos de H1 del gen *PROCR*.

Sorprendentemente, los resultados de los estudios funcionales *in vitro* muestran que el SNP funcional para H1 genera niveles de EPCR disminuidos respecto a H2, lo cual es contrario a lo observado en HUVEC y en los estudios *in silico* realizados utilizando *the Human Splicing Finder*, aunque podría explicar la tendencia a una disminución en los niveles de mRNA a medida que aumenta la presencia de los alelos H1.

4.3. La enfermedad de Behçet y los haplotipos del gen *PROCR*

Puesto que un nivel reducido de APC es un factor independiente tanto de TEV (España F, 2001) como de trombosis arterial (Zorio E, 2006), y además se ha asociado con una alta incidencia de TEV en la enfermedad de Behçet (Navarro S, 2004), hemos estudiado los genotipos del gen *PROCR* presentes en el grupo de pacientes y controles. Independientemente de la presencia de TEV en los pacientes, observamos que la presencia del alelo H3 era menor en pacientes que en controles (OR 0,29; IC95%: 0,12-0,70, *P*=0,006). Tras ajustar por edad, sexo y presencia de defectos trombofílicos (deficiencia de PC, PS o AT, factor V Leiden o protrombina 20210A), no observamos variación en la asociación entre pacientes y controles respecto al alelo H3 (OR 0,19; IC95%: 0,07-0,55, *P*=0,002). Tras excluir a los 19 pacientes que habían sufrido un TEV, no se observaron cambios significativos en las ORs (OR 0,31 y 0,183, respectivamente). Estos resultados indicaban que el alelo H3 protege frente a la enfermedad de Behçet. Por el contrario, la frecuencia del alelo H1 no presentaba diferencias entre pacientes y controles

4.3.1. Los haplotipos del gen PROCR y el riesgo de TEV en pacientes con enfermedad de Behçet

Dado que H1 y H3 del gen *PROCR* han sido asociados con el TEV, estudiamos la asociación de estos haplotipos con el riesgo de sufrir un evento trombótico en los pacientes con la enfermedad de Behçet. La presencia de H1 mostró una tendencia a estar más presente en el subgrupo de pacientes sin trombosis (84%) que en aquellos que habían sufrido trombosis (63%) (*P*=0,056). En la regresión logística, tras ajustar por edad, sexo y presencia de defectos trombofílicos, la presencia del alelo H1 reduce el riesgo de trombosis en los pacientes con Behçet (OR: 0,21; *P*=0,023). En cambio, la presencia del alelo H3 no estaba asociado al riesgo de trombosis en estos pacientes (OR: 1,59; *P*=0,716).

4.3.2. Niveles de APC, sEPCR y la enfermedad de Behçet y la trombosis

Tal como había sido descrito previamente por nuestro grupo (Navarro S, 2004), encontramos que los niveles de APC estaban significativamente disminuidos en los pacientes comparados con los controles sanos (*P*<0,001), mientras que los niveles de fibrinógeno, factor VIII, FVW y proteína C reactiva (PCR) eran significativamente superiores en los pacientes (*P*<0,001). Además, el grupo de pacientes también presentaba niveles de sEPCR más bajos que los controles (*P*<0,001), y estos resultados no cambiaban tras eliminar a los pacientes con trombosis del grupo de pacientes con Behçet.

Tras estratificar los niveles de sEPCR por cuartiles según su distribución en el grupo control, se mostró que la OR para el cuarto cuartil comparado con el primero era de 0,15 (IC 95%: 0,05-0,45). Tras ajustar por edad, sexo y defectos trombofílicos, no se modificó el efecto del sEPCR sobre el Behçet (OR: 0,10; IC 95%: 0,02-0,34; *P*=0,001). Estos resultados muestran que bajos niveles de sEPCR protegen frente a la enfermedad de Behçet.

Dado que los niveles elevados de sEPCR se ha demostrado que están asociados a H3, y hemos visto que este haplotipo protege frente a la enfermedad de Behçet, realizamos un análisis bivariante incluyendo en el modelo el cuarto cuartil de sEPCR y la presencia del alelo H3. Los valores de ORs ajustados fueron de 0,10 (IC 95%: 0,01-0,46; *P*=0,005) y 0,92 (IC 95%: 0,14-5,9; *P*=0,929), respectivamente, lo que sugiere que el

efecto protector del H3 frente a la enfermedad de Behçet es probablemente debido a su asociación con los niveles aumentados de sEPCR.

Se estudió la relación de los niveles de APC y sEPCR en los pacientes y controles según los haplotipos del gen *PROCR*, y se observó que los niveles de APC aumentaban en presencia de los alelos H1, tanto en pacientes como en controles (P<0,001 y P=0,010, respectivamente). Los niveles de sEPCR eran significativamente superiores en los portadores de H3 (P<0,001). Tal como era de esperar, los niveles de APC eran menores en los pacientes que habían sufrido trombosis frente a los pacientes que no la habían sufrido (P=0,026), y no se observaron diferentes significativas respecto a los niveles de sEPCR.

4.3.3. Niveles de APC, sEPCR y uveítis

Tras comparar los niveles de APC en pacientes con y sin afectación ocular, se observó que aquellos con afectación presentaban niveles de APC significativamente más bajos (P=0,001), y esta diferencia se podría atribuir a la uveítis posterior ya que aquellos pacientes que la presentaban tenían niveles significativamente más bajos que aquellos con uveítis anterior (P<0,001). No se observaron diferencias significativas en los niveles de sEPCR respecto a la afectación ocular o uveítis.

4.3.4. H3, los marcadores de inflamación y la PC

Tal como hemos comentado anteriormente, los marcadores de inflamación (α_1-AT, fibrinógeno, factor VIII, FVW y PCR) estaban significativamente elevados en los pacientes con enfermedad de Behçet, y por otro lado, se veía asociación con una menor presencia de H3. Por tanto, para conocer la influencia de dicho haplotipo sobre los niveles de los marcadores de inflamación, se distribuyeron estos niveles en función de la presencia del alelo H3. Tal como era de esperar, los niveles de sEPCR eran superiores en los portadores de H3. Entre los controles, se observó que los portadores de H3 presentaban niveles significativamente elevados de fibrinógeno, factor VIII, FVW y PCR (P<0,034). Entre los pacientes con el alelo H3 existía una tendencia a niveles elevados de marcadores de inflamación pero sin significación estadística.

Por otro lado, y dado que H3 está asociado con niveles de PC elevados, analizamos esta asociación en los grupos de estudio. Los controles sanos portadores de H3 presentaban niveles de PC significativamente más elevados que aquellos que no eran portadores. Entre los pacientes, los portadores de H3 presentaban niveles elevados de PC comparados con los no portadores (128%±10% vs. 115%±24%), aunque las diferencias no eran estadísticamente significativas (*P*=0,149), seguramente debido al bajo número de pacientes portadores de H3.

5. DISCUSIÓN

5.1. Estudio de los polimorfismo del gen *THBD*

Se ha descrito que la presencia del alelo T1418 del gen *THBD* está asociado con una disminución de los niveles de sTM en el plasma de pacientes con TEV y en el medio de cultivo de las HUVEC. Paralelamente, este polimorfismo está asociado a una mayor presencia de TM funcional unida a membrana. Todo ello podría explicar los mayores niveles de APC y menor riesgo de TEV descritos en individuos portadores de este alelo. Los datos muestran que tanto la presencia del alelo 1418T del gen *THBD* como los niveles bajos de sTM son parámetros independientemente asociados con una disminución del riesgo de TEV.

Tras realizar un ajuste del riesgo de TEV en función de la edad (pacientes y controles en dos grupos según edad ≤ 45 años o >45), se observó que el efecto protector de la presencia del alelo 1418T se manifestaba principalmente en el grupo joven. Se necesitarían estudios para confirmar el efecto protector dependiente de la edad.

Además, el estudio de las HUVEC indica que la presencia del alelo 1418T del gen *THBD* se asocia significativamente con más TM unida a membrana y menos sTM en el medio. Paralelamente, se ha demostrado que la capacidad de cofactor de la trombina para activar a la PC de la TM unida a la membrana de las células HUVEC, era mayor en las células portadoras del genotipo TT que en las de genotipo CC ya que las primeras tienen mayor cantidad de TM en membrana. Todo ello, podría explicar los mayores niveles de APC y menor riesgo de TEV descritos en individuos portadores del alelo 1418T.

Dado que no se encontraron diferencias entre los niveles de mRNA según el polimorfismo y por el contrario sí se observó mayor cantidad de TM unida a membranas, se podría sugerir que la mayor cantidad de TM que presenta el alelo 1418T no es debida a una mayor producción de TM sino a que esta TM es más estable y con menor tendencia a ser liberada al plasma en forma de sTM. El alelo 1418T codifica para el aminoácido valina en la posición 455 de la proteína y está localizado cerca del lugar de corte de las proteasas. El cambio de aminoácido de alanina a valina puede inducir una protección frente a la acción de las proteasas.

5.2. Estudio de los haplotipos del gen *PROCR*

En cuanto al estudio de los haplotipos del gen *PROCR*, se han confirmado los estudios previos (Medina P, 2004 & 2005; Pecheniuk NM, 2008) que explican que los individuos con el genotipo H1H1 tienen menor riesgo de TEV, mayores niveles de APC en plasma y valores de sEPCR normales comparados con aquellos que tienen otros genotipos.

Por primera vez, gracias a los cultivos con HUVEC, se ha demostrado que el efecto protector frente al TEV de los portadores de H1 podría deberse a un aumento de EPCR funcional en membrana que consecuentemente conlleva una mayor activación de la PC y niveles ligeramente disminuidos de sEPCR en el medio.

Se ha identificado el SNP g.2532T>C (rs2069948) localizado en el intrón 1 como posible SNP funcional de H1 del gen *PROCR*. En el experimento la presencia de este SNP estaba relacionada con la disminución de la expresión de la proteína, posiblemente debido a un cambio en la eficiencia del *splicing*, lo que podría explicar la discordancia de estos resultados comparados con los resultados obtenidos en HUVEC.

Por el contrario, se ha confirmado que los individuos con el genotipo H3H3 presentan mayor riesgo de padecer TEV y niveles elevados de sEPCR en plasma. En las HUVEC, se ha demostrado que el alelo H3 está asociado con menos EPCR funcional unido a membrana y mayor cantidad de sEPCR en el medio de cultivo y por ello, menor activación de la PC *in vitro*. Esta disminución en la cantidad de EPCR funcional unido a membrana parece no estar relacionada con la expresión génica de *PROCR* dado que los niveles de mRNA completo no varían en función de H3. Los niveles de mRNA truncado sí que muestran un aumento en caso del alelo H3 pero parece improbable que dicho aumento sea el responsable del elevado incremento de sEPCR en los portadores del alelo H3. Por tanto, nuestros datos sugieren que el alelo H3 podría conferir una mayor susceptibilidad al EPCR para ser escindido del endotelio mediante metaloproteasas, dando lugar a niveles significativamente disminuidos en membrana y aumentados en plasma. Este efecto de H3 sobre la proteína se podría explicar por el cambio de aminoácido serina por glicina en la posición 219, que haría más susceptible al EPCR de la acción de la metaloproteasa que lo libera al plasma, como por ejemplo la metaloproteasa ADAM17.

5.3. Estudio sobre los haplotipos del gen *PROCR* y la enfermedad de Behçet

Los resultados hallados en este estudio indican que el H1 reduce el riesgo de sufrir TVE en los pacientes con la enfermedad de Behçet a través del aumento de la APC circulante.

En cuanto a H3, se ha demostrado que protege frente a las manifestaciones clínicas asociadas a la enfermedad de Behçet, y probablemente puede deberse a un aumento en los niveles de sEPCR que están asociados a la presencia de este haplotipo. Estos resultados contradicen los hallazgos presentados con anterioridad (Yalçindağ FN, 2008) en los que observaban niveles más elevados de sEPCR en pacientes que en controles. Esta diferencia podría deberse a una distribución diferente en las frecuencias de los haplotipos H3 de las dos poblaciones de estudio. El mecanismo por el cual H3 protege frente a las manifestaciones clínicas en los pacientes con Behçet no ha sido esclarecido. El hecho de que dicho efecto desaparezca tras el ajuste en función de los niveles de sEPCR podría indicar que el mecanismo de protección de H3 es a través del aumento de sEPCR. La hipótesis del efecto protector del sEPCR se refuerza con los resultados que muestran que los parámetros de inflamación están disminuidos en portadores de H3, pero debido al diseño del estudio, no se ha podido demostrar que niveles bajos de sEPCR en los pacientes reflejen un menor daño endotelial.

Adicionalmente, los resultados muestran que los niveles de APC pueden proteger frente a la uveítis posterior en los pacientes con Behçet, dado que se han hallado niveles significativamente inferiores de APC en los pacientes con uveítis posterior. El mecanismo de protección de la APC en estos casos podría estar relacionado con sus funciones antiinflamatorias.

6. CONCLUSIONES

1. En el estudio caso-control, el alelo 1418T del gen *THBD* se asocia con un menor riesgo de tromboembolismo venoso, posiblemente debido a los mayores niveles de proteína C activada observados en los portadores de este alelo.

2. Niveles elevados de trombomodulina soluble están asociados con un mayor riesgo de tromboembolismo venoso y esta asociación es, al menos en parte, independiente del polimorfismo c.1418C>T del gen *THBD*.

3. En los estudios con HUVEC, el alelo 1418T se asocia con niveles elevados de trombomodulina en los lisados celulares, con una mayor activación de proteína C sobre la célula endotelial, y con reducidos niveles de trombomodulina soluble en el medio de cultivo.

4. En el estudio caso-control, la presencia del genotipo H1H1 del gen *PROCR* reduce significativamente el riesgo de tromboembolismo venoso, mientras que la presencia del genotipo H3H3 está asociada con un aumento de dicho riesgo.

5. En los estudios con HUVEC, los niveles de EPCR en la membrana aumentan a medida que aumenta el número de alelos H1, mientras que los del EPCR soluble disminuyen significativamente. Por el contrario, la presencia del alelo H3 se asocia con niveles disminuidos de EPCR en la membrana y con niveles aumentados de EPCR soluble en el medio de cultivo.

6. De los 4 polimorfismos específicos del haplotipo H1 del gen *PROCR*, el g.2532T>C parece ser el responsable de los efectos de dicho haplotipo.

7. Los niveles de proteína C activada y de EPCR soluble están reducidos en los pacientes con enfermedad de Behçet. Además, el haplotipo H3 protege de la enfermedad de Behçet, posiblemente por su asociación con niveles aumentados de EPCR soluble.

8. La presencia del haplotipo H1 del gen *PROCR* reduce el riesgo de trombosis en los pacientes con enfermedad de Behçet. En cambio, la presencia del alelo H3 no está asociada con el riesgo de trombosis en estos pacientes.

9. Los pacientes con enfermedad de Behçet y afectación ocular tienen niveles de proteína C activada más bajos que aquellos sin afectación. Esta diferencia se

podría atribuir a la uveítis posterior, ya que los pacientes que la presentan tienen niveles significativamente más bajos que aquellos con uveítis anterior.

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8. ANEXO: Abreviaturas

α1-AT: Alfa1- antitripsina

APC: Proteína C activada

AT: Antitrombina

EGF: Factor de crecimiento epidérmico

EPCR: Receptor endotelial de la proteína C

F: Factores

FT: Factor tisular

FVW: Factor Von Willebrand

HUVEC: Células endoteliales de vena umbilical humana

MHC: Complejo mayor de histocompatibilidad

OR: *Odds Ratio*

PAI: Inhibidor del activador del plasminógeno

PAR-1: Receptor activado por proteasas tipo 1

PC: Proteína C

PCI: Inhibidor de la proteína C

PCR: Proteína C reactiva

PS: Proteína S

PT: Protrombina o factor II

R: Receptores

sEPCR: Forma soluble de EPCR

SNP: Polimorfismo de un solo nucleótido

sTM: Forma soluble de TM

TEV: Tromboembolismo venoso

TAFI: Inhibidor de la fibrinólisis activable por trombina

TFPI: inhibidor de la vía del factor tisular

TM: Trombomodulina

tPA: Activador tisular del plasminógeno

uPA: Activador del plasminógeno tipo uroquinasa

Arteriosclerosis, Thrombosis, and Vascular Biology

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Arterioscler Thromb Vasc Biol. 2013;33:1435-1440; originally published online March 21, 2013;
doi: 10.1161/ATVBAHA.113.301360
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Association of the Thrombomodulin Gene c.1418C>T Polymorphism With Thrombomodulin Levels and With Venous Thrombosis Risk

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***Objective*—**To investigate the association of the *THBD* c.1418C>T polymorphism, which encodes for the replacement of Ala455 by Val in thrombomodulin (TM), with venous thromboembolism (VTE), plasma soluble TM, and activated protein C levels. In addition, human umbilical vein endothelial cells (HUVEC) isolated from 100 umbilical cords were used to analyze the relation between this polymorphism and *THBD* mRNA and TM protein expression.

***Approach and Results*—**The *THBD* c.1418C>T polymorphism was genotyped in 1173 patients with VTE and 1262 control subjects. Levels of soluble TM and activated protein C were measured in 414 patients with VTE (not on oral anticoagulants) and 451 controls. HUVECs were genotyped for the polymorphism and analyzed for *THBD* mRNA and TM protein expression and for the ability to enhance protein C activation by thrombin. The 1418T allele frequency was lower in patients than in controls ($P<0.001$), and its presence was associated with a reduced VTE risk, reduced soluble TM levels, and increased circulating activated protein C levels ($P<0.001$). In cultured HUVEC, the 1418T allele did not influence *THBD* expression but was associated with increased TM in cell lysates, increased rate of protein C activation, and reduced soluble TM levels in conditioned medium.

***Conclusions*—**The *THBD* 1418T allele is associated with lower soluble TM, both in plasma and in HUVEC-conditioned medium, and with an increase in functional membrane–bound TM in HUVEC, which could explain the increased activated protein C levels and the reduced VTE risk observed in individuals carrying this allele. **(*Arterioscler Thromb Vasc Biol*. 2013;33:1435-1440.)**

Key Words: endothelial cells ■ gene expression ■ protein C ■ thrombomodulin ■ venous thrombosis

Venous thromboembolism (VTE) has a significant hereditary component,[1] and some reports have assessed the genetic components of plasma variability in hemostasis-related phenotypes through family-based sampling designs.[2,3] Heritabilities range from 0.11 to 0.83[1]

The protein C (PC) anticoagulant pathway plays an important role in regulating thrombin generation and inflammatory reactions. PC circulates in plasma as a zymogen, which is activated on the surface of endothelial cells by the thrombin–thrombomodulin (TM)–endothelial cell PC receptor (EPCR) complex.[4] Once activated, PC is a potent anticoagulant and anti-inflammatory protease.[5–7]

TM is an endothelial cell membrane protein that acts as a cofactor for thrombin in the activation of PC. TM is also independently involved in cytoprotective responses.[6–9] Studies in animal models suggest that TM deficiency is associated with a prethrombotic state.[10–12] Heterogeneous soluble forms of TM (sTM) circulate in plasma,[13] and their levels are increased in several clinical conditions.[14] Some of these soluble forms might have anticoagulant activity.[15] Normal levels vary according to the assay used for the measurements, ranging from 2.7 to 5.4 ng/mL[16] or from 25 to 65 ng/mL.[17]

Genetic studies have identified many mutations and polymorphisms in the *THBD* gene, but no clear association with venous or arterial thrombosis has been found.[18] A common single-nucleotide polymorphism in the coding region of *THBD* (c.1418C>T) (rs1042579), which results in the replacement of Ala455 by Val, has been described.[19] This dimorphism is located in the TM region responsible for thrombin binding and PC activation, suggesting a potential role in the modulation

Received on: November 9, 2012; final version accepted on: March 8, 2013.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.301360/-/DC1.

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of TM function. However, its association with venous[19–23] and arterial thrombosis[24–26] is not consistent.

In the present study, we investigated the association of this polymorphism with VTE and plasma TM and activated PC (APC) levels. In addition, human umbilical vein endothelial cells (HUVEC) isolated from 100 umbilical cords were used to analyze the relation between this polymorphism and *THBD* mRNA and TM protein expression. We found that the presence of the *THBD* 1418T allele, which encodes for TM Val455, is associated with a decrease in sTM, both in plasma and in HUVEC-conditioned medium, and an increase in functional membrane–bound TM, which could explain the increased levels of circulating APC and the reduced risk of VTE in individuals carrying this allele.

Materials and Methods

Study design, experimental methods, and statistical analyses are described in detail in the online-only Supplement.

Our study included 1173 unrelated white patients with VTE and 1262 unrelated healthy subjects with no history of thromboembolic disease.

HUVEC from 100 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks. TM levels were measured in HUVEC-conditioned medium and in cell lysates. For Western blot analysis, cell lysates (1.0 μg per lane) from representative HUVEC carrying the 1418 CC (n=2), CT (n=2), and TT (n=2) genotype were subjected from 4% to 12% SDS-polyacrylamid gel electrophoresis (PAGE) under reducing conditions and transferred to a polyvinylidene difluoride membrane. PC activation on HUVEC was performed by incubating cells with PC and bovine thrombin, in the presence or absence of a rat monoclonal anti-EPCR antibody, followed by the measurement of the amidolytic activity of the APC formed.

Genotyping of the *THBD* c.1418C>T polymorphism was performed by direct sequencing and quantification of mRNA transcripts by real time–quantitative polymerase chain reaction using semiautomatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany).

Results

Clinical Characteristics of the Subjects

The clinical characteristics of the study subjects are presented in Table 1. These features did not significantly differ in the samples from the 3 hospitals (data not shown). About 32% of the patients (375 of 1173) had spontaneous thrombosis, which is defined as thrombosis in the absence of known triggering risk factors (use of contraceptives, pregnancy, surgery, trauma, and immobilization). The prevalence of classical prothrombotic polymorphisms was similar to that described in other series.

THBD c.1418C>T Polymorphism and VTE Risk

The 1173 patients and 1262 control subjects were successfully genotyped for the *THBD* c.1418C>T polymorphism. Table 2 shows the genotype distribution. Among the 1262 healthy subjects, the frequency of the 1418T allele was 0.175, which is similar to that reported in previous studies (0.180,[19] 0.169,[23] and 0.184[27]) but lower than in other studies (0.261[20] and 0.280[25]). Given that the control subjects were recruited in 3 different geographical areas of Spain, we analyzed the 1418T allele frequency in the 3 control populations. The frequencies of the 1418T allele were 0.184, 0.172, and 0.169 in the control groups from Valencia, Murcia, and Salamanca, respectively, with no significant differences between them. Among the 1173 patients with VTE, the frequency of the 1418T allele was 0.136 (0.140, 0.136, and 0.125 in the group of patients from Valencia, Murcia, and Salamanca, respectively, with no significant differences among them). This is not so different from that observed in a previous study (0.154[19]) but lower than that reported in other studies (0.213,[20] 0.196,[23] and 0.180[25]).

Table 1. Characteristics of the Study Subjects

	Patients	Controls	Statistical Significance *P*
n	1173	1262	
Age: median (10th–90th percentile)	45 (34–57)	44 (33–57)	*P*=0.389
Age at first onset: median	42 (31–54)	N.A.	
Men, n (%)	613 (52)	657 (52)	*P*=0.954
Pulmonary embolism, n (%)	267 (23)	N.A.	
Recurrent thrombosis, n (%)	281 (24)	N.A.	
Family thrombosis, %	258 (22)	N.A.	
Spontaneous thrombosis, n (%) *	375 (32)	N.A.	
Factor V Leiden, n (%)			
–/–	997 (85)	1229 (97)	
+/– and +/+	173 and 3 (15)	33 and 0 (3)	*P*<0.001
PT G20210A, n (%)			
GG	1060 (90)	1213 (96)	
GA and AA	105 and 8 (10)	49 and 0 (4)	*P*<0.001

PT indicates prothrombin; and N.A., not applicable.

*After excluding circumstantial risk factors (pregnancy, trauma, surgery, immobilization, and oral contraceptives).

To identify the associations between genotypes and VTE, we performed logistic regression analyses (Table 2). In a univariate analysis, the presence of the 1418T allele or the TT genotype significantly reduced the risk of VTE. Adjustment for sex, age, and the presence of thrombophilic defects in a multivariate analysis did not significantly modify the odds ratio (OR). When the analyses were performed in the subgroups of patients with rethrombosis (adjusted OR for TT: 0.41, 0.14–1.20) or without rethrombosis (adjusted OR for TT: 0.56, 0.30–1.05), and in the subgroup of patients with provoked risk factors (0.60, 0.26–1.24) or without provoked risk factors (0.48, 0.25–0.92), the ORs did not significantly change.

When both patients and controls were grouped into 2 subgroups, according to whether they were >45 years or not, the protective effect of the 1418T allele was even more pronounced in the younger individuals (adjusted OR for CT and TT: 0.55, 0.43–0.71), whereas the protective effect of the 1418T allele disappeared in those subjects aged >45 years (1.06, 0.81–1.39).

sTM Levels and VTE Risk

To investigate whether sTM levels associated with the risk of VTE, we compared sTM levels in 414 patients with VTE (excluding those undergoing coumarin therapy) and in 451

Table 2. Genotype and Allele Distribution of *THBD* Gene c.1418C>T Polymorphism in 1173 Patients and 1262 Control Subjects

			OR (95% CI)	
THBD c.1418C>T Genotype	Cases, n (%)	Controls, n (%)	Crude	Adjusted*
Overall				
CC	875 (74.6)	857 (67.9)	1†	1†
CT	277 (23.6)	368 (29.2)	0.74 (0.61–0.88)	0.77 (0.63–0.93)
TT	21 (1.8)	37 (2.9)	0.56 (0.32–0.96)	0.55 (0.31–0.98)
CT and TT	298 (25.4)	405 (32.1)	0.72 (0.60–0.86)	0.75 (0.62–0.90)
T allele	319 (13.6)	442 (17.5)	0.74 (0.63–0.87)	-

CI indicates confidence interval; and OR, odds ratio.

*Adjusted for age, sex, and presence of thrombophilic defects (factor V Leiden, prothrombin 20210A, protein C deficiency, protein S deficiency, and antithrombin deficiency).

†Reference group.

healthy individuals from whom plasma was available. sTM was significantly higher in the 414 patients with VTE than in the 451 healthy individuals (P=0.005; Table 3). To study whether the sTM plasma levels were influenced by the presence of the *THBD* 1418T allele, we also calculated these levels according to the genotype, in overall individuals as well as in the subgroups of individuals aged <46 years or >45 years (Table 3). In both patients and controls, sTM levels significantly decreased when the number of 1418T alleles increased (P<0.001).

We also analyzed the distribution of sTM levels in patients and controls according to sex. Among the patients with VTE, sTM was higher in men (4.18, 3.75–4.61) than in women (3.88, 3.39–4.68; P=0.001). Also in controls, sTM was higher in men (4.09, 3.42–4.76) than in women (3.72, 3.3–4.23; P<0.001). These results are in agreement with those in previous studies.[16,28]

To assess whether the sTM levels were associated with an increased risk of VTE, we distributed sTM levels into quartiles, as measured in the healthy control group. By taking the first quartile as the reference group, the ORs for the second, third, and fourth quartiles were 1.23 (0.81–1.87), 1.72 (1.16–2.56), and 1.75 (1.18–2.60), respectively, after adjusting for age, sex, and the presence of thrombophilic defects (P for trend <0.001).

Because sTM levels are apparently associated with the *THBD* c.1418C>T polymorphism, we performed a multivariate analysis to include age, sex, presence of thrombophilic defects, sTM<3.86 ng/mL (50th percentile of the distribution among controls), and the presence of the 1418T allele in the model. The ORs for the presence of the 1418T allele (CT and TT carriers) were 0.75 (0.62–0.90) in the absence and 0.61 (0.45–0.84) in the presence of sTM<3.86 in the model. The OR for sTM<3.86 was 0.65 (0.49–0.86) when the 1418T allele was not present in the model, and 0.61 (0.45–0.84) when the 1418T allele was present, suggesting that both parameters independently protect against VTE.

Activated PC

Given that one possible mechanism by which the carriers of the 1418TT genotype have a decreased risk of VTE is the presence of increased plasma APC levels, we also determined the plasma levels of APC in 414 patients with VTE (excluding those undergoing coumarin therapy) and 451 healthy individuals in whom sTM was measured (Table 3). As previously reported,[29] the level of circulating APC was lower in patients

Table 3. Levels of sTM and APC (Median and 25th–75th Percentile) in 414 Patients (Without Coumarin Therapy) and 451 Controls According to the *THBD* gene c.1418C>T Polymorphism

c.1418C>T Genotype	All Individuals	CC Carriers	CT Carriers	TT Carriers	P
sTM, ng/mL					
Patients	4.06 (3.56–4.67)	4.18 (3.70–4.86)	3.77 (3.48–4.11)	3.28 (2.79–3.57)	<0.001*
	(n=414)	(n=319)	(n=86)	(n=9)	
Controls	3.86 (3.38–4.48)	4.05 (3.51–4.68)	3.68 (3.21–4.26)	2.99 (2.60–3.45)	<0.001*
	(n=451)	(n=293)	(n=138)	(n=20)	
	0.005†	0.008†	0.435†	0.517† ←	
APC, ng/mL					
Patients	0.98 (0.72–1.22)	0.97 (0.72–1.23)	0.97 (0.72–1.17)	1.23 (0.79–1.41)	0.386*
	(n=414)	(n=319)	(n=86)	(n=9)	
Controls	1.20 (0.98–1.49)	1.19 (0.95–1.47)	1.22 (1.00–1.61)	1.29 (1.13–1.54)	0.191*
	(n=451)	(n=293)	(n=138)	(n=20)	
	<0.001†	<0.001†	<0.001†	0.724† ←	

APC indicates activated protein C; and sTM, soluble thrombomodulin.

*Kruskal–Wallis Test; †Mann-Whitney *U* test.

with VTE (0.98; 0.72–1.22) than in controls (1.20; 0.98–1.49; $P<0.001$). We also distributed the APC levels according to the *THBD* c.1418C>T genotype. In both patients and controls, there was a trend to higher APC levels when the number of 1418T alleles present increased, although (given the low number of subjects carrying the TT genotype) these differences were not significant.

An inverse correlation was observed between the APC and sTM levels among controls ($r= -0.161$; $P=0.001$).

TM and mRNA Levels in HUVEC

To further investigate whether the *THBD* c.1418C>T polymorphism is functional, we analyzed the HUVECs derived from 100 human umbilical cords from white newborns. Three cultures were 1418TT, 26 were 1418CT, and 71 were 1418CC. In these HUVECs, we determined the amount of TM in culture medium (sTM) by ELISA and in the cell lysates (membrane-bound TM) by ELISA and Western blot. TM levels in the cell lysate increased ($P<0.001$) and the level of sTM in the cultured medium decreased ($P<0.001$) in parallel with the increase of the number of 1418T alleles present (Figure 1). Western blot data confirmed the results obtained by ELISA as follows: the concentration of TM increased when the number of 1418T alleles increased ($P<0.001$; Figure 2).

We also determined the *THBD* mRNA levels in 95 HUVECs in which mRNA was available. *THBD* mRNA levels in the 67 HUVECs carrying the 1418CC genotype (7.5, 4.1–8.9) did not significantly differ from those found in the 26 HUVECs with the 1418CT genotype (6.1, 3.6–6.6) and in the 2 HUVECs with the 1418TT genotype (6.8 and 3.0; $P=0.496$).

PC Activation on HUVEC

PC activation on HUVEC carrying the 1418CC, CT, and TT genotypes was compared (Figure 3). Because 2 haplotypes of the *PROCR*, H1 and H3, have been associated with the rate of activation of PC in cultured HUVEC[30] and with the levels of APC in human plasma,[28,31] we selected HUVEC with the same *PROCR* genotype (H1H2). The rate of PC activation

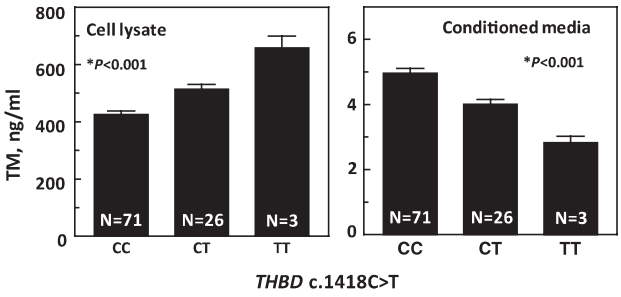

Figure 1. Thrombomodulin (TM) levels in conditioned medium (CM) and cell lysates from human umbilical vein endothelial cells (HUVEC), according to their c.1418C>T genotype. HUVEC were grown from 80% to 100% confluence and exposed for 3 hours to free serum-CM (50 μL/well), as indicated in the online-only Data Supplement. Thereafter, CM was stored at −72 °C, and cells were lysed with 50 μL/well of lysis buffer. Levels of TM in CM and cell lysates were measured as indicated in the online-only Data Supplement. Data are expressed as mean±SEM; *Kruskal–Wallis test.

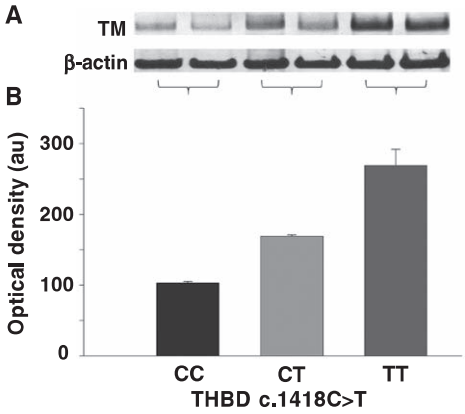

Figure 2. Western blot analysis of human umbilical vein endothelial cells (HUVEC) thrombomodulin (TM) expression according to the *THBD* gene c.1418C>T polymorphism. **A**, Cell lysates were prepared as indicated in the online-only Data Supplement. One microgram of representative lysates from 2 CC, 2 CT, and 2 TT HUVECs was electrophoresed on a 4% to 12% gradient SDS-polyacrylamide gel, electrobotted onto polyvinylidene difluoride membranes, and stained with anti-TM and anti β-actin antibodies. **B**, TM protein in arbitrary optical units (AU) expressed as mean±SD of 2 different HUVECs of each genotype. Values were normalized by β-actin and taking the 1418CC genotype as 100, $P<0.001$ (Kruskal–Wallis test).

increased in parallel with the increase of the number of 1418T alleles present. Thus, the activation of PC on HUVEC CC was ≈60% of the activation on HUVEC TT. To estimate the amount of PC activation that is attributable to TM in the absence of EPCR, HUVECs were incubated with mAb RCR-379 to block EPCR-dependent PC activation. Incubation with the anti-EPCR antibody drastically reduced PC activation, but residual PC activation still was higher on HUVEC TT than on HUVEC CC (Figure 3).

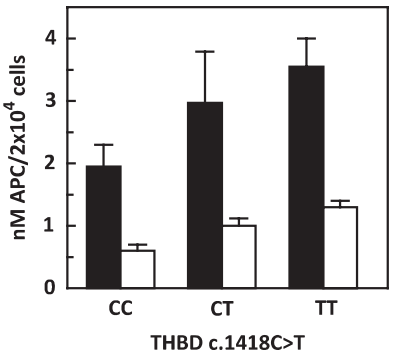

Figure 3. The rate of protein C activation increases with the increase in the number of *THBD* 1418T alleles present. Human umbilical vein endothelial cells (HUVEC) from 2 CC, 2 CT, and 2 TT genotype were incubated with protein C and thrombin at 37°C for half-an-hour as described in the online-only Data Supplement. The amount of activated protein C (APC) formed/2×10^4 cells is plotted against the genotype indicated. The results represent the mean±SD of 2 separate experiments performed in duplicate. Solid bars represent protein C activation in the absence of anti-endothelial cell protein C receptor (EPCR) mAb, RCR-379. Open bars represent protein C activation in the presence of anti-EPCR mAb, RCR-379. In the absence of cells, the rate of protein C activation was ≈0.2 nmol/L APC.

Discussion

In the present study, we observed that carriers of the 1418T allele in the *THBD* gene show a lower risk of VTE than noncarriers. We also found that the risk of VTE increased at increasing levels of plasma sTM, and that the presence of the 1418T allele and low sTM levels was independently associated with a decrease in the risk of VTE. Furthermore, we showed that HUVEC carrying the 1418T allele had significantly higher membrane–bound TM and lower sTM levels in culture medium than those not carrying, whereas the *THBD* mRNA level was similar in carriers and noncarriers. Finally, we showed that cellular TM activity, defined as the thrombin-mediated PC activation on intact HUVEC, was also higher in TT cells than in CC cells.

Several studies have investigated the association between the *THBD* c.1418C>T polymorphism and venous and arterial thrombosis, with conflicting results. Some studies did not find an association between the polymorphism and the risk of VTE,[22,23] although 1 study observed a lower risk in blacks carrying the 1418T allele.[22] One study observed a higher risk in the presence of the 1418T allele.[32] Regarding the risk of arterial thrombosis, 1 study found a protective effect for the T allele,[25] whereas another observed an increased risk associated with the presence of the T allele.[27] Wu et al[26] reported that the 1418T allele increased the risk of coronary heart disease in blacks, but not in whites. An explanation for these discrepancies may lie in differences in study designs and patient populations, and in small sample sizes. In fact, the sample size in the majority of the previous reports ranged from 18 to 302 patients with VTE, whereas in most studies the age of patients and controls was quite higher than in our study. To further explore the possibility that age could explain the observed differences, we analyzed the association of the c.1418C>T polymorphism with the risk of VTE after classifying patients and controls in 2 subgroups using a cutoff point of 45 years. The adjusted OR for CT and TT was 0.55 (0.43–0.71) in individuals aged ≤45 years and 1.06 (0.81–1.39) in individuals aged >45 years. This might indicate that the protective effect of the 1418T allele against VTE is mainly manifested in younger people. Further studies are needed to confirm and analyze this age-related effect in more detail.

The mechanism by which the 1418T allele protects against VTE is not clear. One explanation could be that the Val455 form has higher cofactor activity for PC activation. However, recombinant Val455 and Ala455, produced by Cos-1 cells, were found equally active in PC activation.[33] Another explanation could be that the 1418T allele is associated with increased membrane-bound TM, resulting in increased PC activation. In fact, the present study shows that the 1418T allele is associated with increased amount of TM in cell lysates and with decreased sTM levels in both plasma and HUVEC-cultured medium. This study also shows that the increased amount of TM in the cell lysates is associated with increased PC activation, which would result in the subsequent increase in circulating APC, as observed in the present study in the plasma of both control subjects and patients with VTE, and a decrease in the risk of VTE.[29] Because there were no significant differences in *THBD* mRNA levels according to the c.1418C>T polymorphism in HUVEC, together these results suggest that the membrane-bound TM carrying the 455Val (1418T allele) might be more stable and less prone to shedding. Soluble TM represents cleaved forms of membrane-bound TM with loss of part of the serine–threonine rich region, the transmembrane domain, and the cytoplasmic tail.[34] The 455Val residue is located not far from the presumed cleavage site and may induce a protection from TM cleavage by proteases.

Alternatively, the observed association between the *THBD* c.1418C>T polymorphism and the risk of VTE could be attributed to another polymorphism in tight linkage disequilibrium with c.1418C>T. In fact, we previously observed complete linkage disequilibrium (r^2=0.98) between the c.1418C>T and c.2729A>C (rs3176123) polymorphisms, and a moderate linkage disequilibrium between the c.1418C>T and c.–1208-1209delTT (r^2=0.27).[35]

As to the levels of sTM, our results indicate that increased levels are associated with higher VTE risk, and this association is, at least in part, independent of the *THBD* c.1418C>T polymorphism. Increased sTM levels have also been reported in patients with recurrent VTE.[36] In contrast, 1 report did not find association between sTM and risk of VTE.[22]

In conclusion, this work provides new data on the age-dependent association between the presence of the 1418T allele in the *THBD* gene and a lower VTE risk. Our results suggest that the 1418T allele in the *THBD* gene may produce a more stable TM that is protected from shedding, which will result in higher PC activation rate and a lower risk of VTE.

Acknowledgments

We thank Marta Gurrea, Silvana Aniorte, Eugenia Romaguera, and Elisabet Rodríguez for collecting the umbilical cords, and Úrsula Salinas and Josefa Llorens for technical assistance.

Sources of Funding

This work was supported by the PN de I+D+I 2008–20011 (ISCIII PS09/00610), FEDER and Redes RECAVA (RD06/0014/0004 and RD06/0014/0039), Cardiovascular (RD/12/0042/0029), and REDISCOR (RD06/0003/1001), and by Consellería de Educación, Formación y Empleo, Generalitat Valenciana (PROMETEO 2011/027), Spain.

Disclosures

None.

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Significance

Thrombomodulin (TM) is an important cofactor for the activation of protein C by thrombin on the endothelial cell membrane. In animal models, TM deficiency has been associated with thrombosis risk. Several variants of the human *THBD* gene have been reported, but their association with thrombosis is not conclusive. One of these is the *THBD* c.1418C>T polymorphism. In the present study, we demonstrated that in cultured human umbilical vein endothelial cells the *THBD* 1418T allele is associated with increased levels of functionally active membrane–bound TM and lower levels of TM in the human umbilical vein endothelial cells-conditioned medium. In concordance, individuals with the *THBD* 1418T allele showed lower plasma levels of soluble TM, higher plasma levels of circulating activated protein C, and a reduced risk of venous thromboembolism. Together, these data support the view that the *THBD* c.1418C>T polymorphism is functional and that the 1418T allele protects from venous thromboembolism. Identification of new genetic variants associated with thrombosis will contribute to future strategies to estimate the thrombosis risk of an individual.

Supplemental Materials

Supplementary Methods:

Patients and controls. Our study included 1173 unrelated Caucasian patients with VTE. Patients with an objectively confirmed episode of VTE who consecutively entered the anticoagulation clinics from 3 Spanish hospitals in a time window of 2-7 years were enrolled. Objective diagnoses were made by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan and, when necessary, phlebography or pulmonary angiography. Those patients with known malignant disorders were excluded. The control group included 1262 unrelated healthy subjects with no history of thromboembolic disease. Controls were randomly and prospectively selected to match cases by age, gender and geographic distribution.

All subjects gave their informed consent to enter the study, which was approved by the Ethics Committees of each participating institution, and was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Blood collection. Blood was collected at least 6 month after the acute event. For the thrombophilic study and the determination of sTM, blood was collected in vacuum tubes containing 0.129 M trisodium citrate. For APC determination, blood was collected as indicated before.[1,2] Blood tubes were centrifuged at 1500 x g for 30 min at 4 ºC. Plasma was frozen and stored at -72 ºC. Tubes containing K3EDTA were used for DNA studies.

Plasma thrombomodulin and APC measurement. Levels of plasma sTM were measured with the Imubind Thrombomodulin ELISA kit (American Diagnostica, Stamford, CT). The intra- and inter-assay coefficients of variation were 4.5% and 5.8%, respectively, and the lower limit of detection was 0.4 ng/ml. TM in the HUVEC-CM and in the cell lysates was measured using the Asserachrom TM kit (Diagnostica Stago, Asniéres-sur-Seine, France). The intra- and inter-assay coefficients of variation were 3.6% and 5.1%, respectively, and the lower limit of detection was 1.0 ng/ml. Circulating APC was determined as previously reported.[1,2]

Isolation of HUVEC. HUVEC from 100 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks precoated with endothelial cell attachment factor (Sigma-Aldrich Co, Germany), in medium M199 1X (+) Earle´s, 2mM L-glutamine, 25 mM HEPES, L-aminoacids (Gibco, Life Technologies, Paisley, UK), supplemented with 20% fetal bovine serum (FBS) (Cultek, Madrid, Spain), 1% endothelial cell growth factor (ECGF) (Sigma-Aldrich Co, Germany), 1 mM sodium pyruvate (Gibco), 50 U/ml penicillin, and 50 µg/ml streptomycin sulphate (Gibco) in an atmosphere of 95% air-5% CO_2. Confluent endothelial cell monolayers were harvested from the culture flasks with 0.25% trypsin/EDTA solution/0.02% PBS (Biochrom, Cambridge, UK). Cells were used within two passages. Cells to be used for total RNA isolation were stored at –72 ºC after adding 5 volumes of RNA*later*^{TM} (Ambion, Austin, TX).

Thrombomodulin measurements in HUVEC. For TM determination in HUVEC-CM and cell lysates, cells were plated in 96-well culture plates, at a density of approximately

$20x10^3$ cells/well, and grown to reach 80-100% confluence in the aforementioned CM. Confluent subcultures of HUVEC were incubated for 3 hours with 50 µl of CS-C medium without serum for endothelial cell lines (Sigma-Aldrich Co), supplemented with 2% FBS, 1% ECGF, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin sulphate. After the incubation, the conditioned medium was stored at -72 ºC and cells were washed three times with PBS. For the measurement of TM in cell lysates, washed cells were stored overnight at -80ºC and then treated with 50 µl of lysis buffer pH 7.4 per well (20 mM Tris, 6 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1 mM phenylmethylsulphonyl fluoride), during 2 hours with constant shaking, at 4 ºC.

Western blot detection of thrombomodulin. For Western blot analysis, cell lysates (1.0 µg per lane) from representative HUVEC carrying the CC (N=2), CT (N=2) and TT (N=2) genotypes were subjected to 4-12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and transferred to a PVDF membrane. The membrane was blocked for 1 h at room temperature with 1% BSA in Tris-buffer solution containing 0.05% Tween 20 and then incubated for 2 h with a primary antibody in the same buffer at room temperature. The primary detection antibody used was a rabbit monoclonal anti-TM antibody (1/100) (Abcam, Cambridge, UK). A monoclonal anti-beta-actin antibody (1/500) (Sigma-Aldrich Co, Missouri, USA) was used as loading control of the blots. Then, the bands were visualized using an acid phosphatase conjugated secondary antibody and the nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl phasphate (NBT/BCIP, Sigma) substrate system. Finally, the bands were digitalized using an image analyser (DNR Bio-Imaging Systems, Israel) and quantified by the Gel Capture (v.4.30) and the TotalLab TL-100 (v.2008) software.

Protein C activation on HUVEC. Confluent HUVEC in 96-well plates were washed two times with 50 mM Tris-HCI, pH 7.5, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 100 mM NaCl, 1% BSA (buffer A). The activation of human PC was initiated by the addition of 100 nM human PC and 2 U/ml bovine thrombin (final concentrations) in a total volume of 50 µl. After 30 min at 37 ºC, the reactions were stopped by addition of 50 µl of hirudin (50 U/ml). 75-µl aliquots of the supernatants were transferred into a 96-well microplate and amidolytic activity of APC was determined by adding 15 µl of 8 mM S-2366 in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. The concentration of APC was determined by comparison to a standard curve of amidolytic activity vs. APC concentration constructed with fully activated PC. Under the conditions used in this study, <10% of the PC was activated during the assay. All measurements were performed in duplicate. Where indicated, 50 µg/ml of rat monoclonal anti-EPCR antibody RCR-379 (Abcam, Cambridge, UK) was added to the cells 15 min prior to the addition of PC and thrombin, after which cells were washed. This antibody blocks the ability of EPCR to enhance PC activation by TM.

Genotyping of the THBD c.1418C>T polymorphism. Genomic DNA was isolated from plasma and HUVEC using the Wizard Genomic DNA purification kit (Promega, Madison, WI), following the manufacturer's instructions. The *THBD* c.1418C>T polymorphism (rs1042579) was analyzed by direct sequencing with the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA), using the following set of primers: forward 5´-GTGGCTTCGAGTGCCACTGC-3´ and reverse 5´-CGCACTTGTACTCCATCTTGGCCCTG-3´. The reaction mixture contained 3 µl of 7 ng/µl DNA, 10 µl of 5X colorless Go Taq®Flexi buffer (Promega), 3 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM/each), 0.3 µl of 10 pmol/µl forward primer, 0.135 µl of

1485 ng/µl reverse primer, 0.25 µl of 5 U/µl Taq DNA polymerase (Promega) and 40.27 µl of dH_2O. The reaction mixture was incubated at 95ºC for 4 minutes, followed by 33 cycles of 95ºC for 45 s, 66ºC for 45 s and 72ºC for 45 s, with a final extension of 4 min at 72 ºC. *THBD* gene numbering is according to GenBank Accession ID NM_000361.

mRNA isolation and real-time quantitative PCR analysis. Total RNA was isolated from HUVEC using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and genomic DNA was digested using 1 U/µl of DNase I Amp grade (Invitrogen), according to the manufacturer´s instructions. RNA concentration was measured at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). First strand cDNA was synthesized from the reverse transcription of 1 µg of total RNA in a final reaction volume of 20 µl using the SuperScript III RT and Oligo$(dT)_{12\text{-}18}$ (Invitrogen). Samples were incubated at 65ºC for 5 minutes, and cooled for 1 minute on ice. Then, the reaction mixture was incubated with 4 µl 5X First-Strand Buffer, 1 µl DTT 0.1 M, 1 µl RNaseOUT (40 U/µl) (Invitrogen, Paisley, UK) and 1 µl SuperScript III RT (200 U/µl) at 50ºC for 30-60 minutes. Reverse transcriptase was inactivated by heating for 15 minutes at 70ºC then cooling for 5 minutes on ice. Finally, 1 µl RNase H (2 U/µl) (Invitrogen) was added, and the mixture was incubated at 37ºC for 20 minutes. cDNA was stored at -20ºC. Quantification of mRNA transcripts was performed following the previously reported protocol.[3] The primer sequences were: forward 5´-TAA CGA AGA CAC AGA CTG CGA TT-3´ and reverse 5´-CTA GCC CAC GAG GTC AAG GT-3´. After testing several housekeeping genes, the results were normalized using the TATA-binding protein (TBP) transcripts as best control. RT-qPCR was carried out using a semi-automatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany). Each 15 µl reaction contained 2 µl of 1/10 diluted cDNA, 1.5 µl of 10X LightCycler® FastStart DNA Master Mix, 1.5 µl of 25 mM $MgCl_2$, 0.188 µl of 2 U/µl UNG (only for *THBD* qPCR), 1.5 µl of 10 µM *THBD* primer mix and 0.75 µl *TBP* primers mix (6 pmol/µl each) and 7.1 µl of nuclease-free H_2O. The amplification reaction for the *THBD* gene was initially incubated at 40ºC for 10 minutes, followed by 95ºC for 10 min, and 40 cycles of 5 seconds at 95ºC, 10 seconds at 60ºC, and 5 seconds at 72ºC.

Statistical analysis. The linkage disequilibrium between polymorphisms was analyzed with the HaploView program. Other statistical analyses were conducted using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago, IL). Data are reported as medians and interquartile ranges (25th - 75th percentiles) or means±SEM. Allele frequencies were calculated by gene counting. The Chi-squared test was used to compare percentages. Parameter levels were compared with the Mann-Whitney U-test or the Kruskal-Wallis One ANOVA test. Correlations were assessed by the Spearman test. Logistic regression analysis was done to identify the associations between genotypes and VTE risk. Multivariate analysis was performed using multiple logistic regression by including all the significant covariates in a single step. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistic model. Any differences with a 2-tailed *P* value of <0.05 were considered statistically significant.

Supplementary References:

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Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2014;34:684-690; originally published online January 16, 2014;
doi: 10.1161/ATVBAHA.113.302518
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Functional Analysis of Two Haplotypes of the Human Endothelial Protein C Receptor Gene

P. Medina,* S. Navarro,* E. Bonet, L. Martos, A. Estellés, R.M. Bertina, H.L. Vos, F. España

Objective—To confirm the effect of the endothelial protein receptor gene (*PROCR*) haplotypes H1 and H3 on venous thromboembolism (VTE), to study their effect on endothelial protein C receptor (EPCR) expression in human umbilical vein endothelial cells, and to investigate the functionality of H1 tagging single-nucleotide polymorphisms in an in vitro model.

Approach and Results—Protein C (PC), activated PC, and soluble EPCR (sEPCR) levels were measured in 702 patients with VTE and 518 healthy individuals. All subjects were genotyped for *PROCR* H1 and H3. Human umbilical vein endothelial cells isolated from 111 umbilical cords were used to study the relation between *PROCR* haplotypes, *PROCR* mRNA, cellular distribution of EPCR, and rate of PC activation. Finally, the functionality of the intragenic *PROCR* H1 single-nucleotide polymorphisms was analyzed using a luciferase-based method. We confirmed that individuals carrying H1 have reduced VTE risk, increased plasma activated PC levels, and reduced plasma sEPCR levels and that individuals with the H3H3 genotype have an increased VTE risk and increased plasma sEPCR levels. In cultured human umbilical vein endothelial cells, H1 is associated with increased membrane-bound EPCR, increased rate of PC activation, and reduced sEPCR in conditioned medium, but does not significantly influence *PROCR* mRNA levels. In contrast, H3 is associated with reduced membrane-bound EPCR and increased sEPCR in human umbilical vein endothelial cell–conditioned medium, higher levels of a truncated mRNA isoform, and a lower rate of PC activation. Finally, we identified the g.2132T>C single-nucleotide polymorphism in intron 1 as an intragenic H1-specific functional single-nucleotide polymorphism.

Conclusions—These results support a protective role of *PROCR* H1 against VTE and an increased risk of VTE associated with the H3 haplotype. **(*Arterioscler Thromb Vasc Biol*. 2014;34:684-690.)**

Key Words: gene expression ■ human umbilical vein endothelial cells ■ receptors, cell surface ■ venous thromboembolism

The endothelial protein C receptor (EPCR) is a key component of the protein C (PC) anticoagulant pathway. It is mainly expressed on the surface of endothelial cells, and it increases the rate of PC activation by the thrombin–thrombomodulin complex up to 20-fold.[1] EPCR also modulates the cytoprotective properties of activated PC (APC) in various inflammatory disorders.[2,3]

The EPCR gene (*PROCR*) spans 6 kb and consists of 4 exons.[4] The mature protein comprises 221 amino acids, including an N-terminal extracellular domain, a 25-aa transmembrane domain, and a 3-aa intracytoplasmic sequence. In vivo experiments have demonstrated the importance of EPCR in normal embryonic development; in *PROCR* knockout mice, fibrin deposition around trophoblast giant cells results in thrombosis at the maternal-embryonic interface.[5] Therefore, we hypothesized that DNA variations in *PROCR* that influence protein expression (levels) and function may be relevant. A soluble form of EPCR (sEPCR), which lacks the transmembrane domain and cytoplasmic tail, is present in human plasma,[6] possibly generated through proteolytic cleavage by metalloproteinase activity.[7]

Four common haplotypes of *PROCR* have been reported,[8–10] 3 of which contain ≥1 single-nucleotide polymorphisms (SNPs) that are haplotype specific (H1, H3, and H4). H2 contains the common allele of all SNPs (Figure 1). H1, which is tagged by the minor allele of g.4678G>C (rs9574), has been associated with increased plasma APC levels[8,11–13] and in some, but not all, studies with a reduced risk of venous thromboembolism (VTE).[8–10,12,14,15] A possible explanation for a protective effect of *PROCR* H1 against VTE would be the association with increased plasma APC levels. In fact, it has been reported that a low plasma level of APC, that is, in the absence of overt coagulation, is a strong risk factor for VTE.[16,17] Presently, it is not known which SNP in *PROCR* H1 is responsible for the reported protective effect. H1 contains ≥10 intragenic haplotype-specific SNPs, and any of these might be responsible for the observed effects.

Received on: September 12, 2013; final version accepted on: January 6, 2014.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302518/-/DC1.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.113.302518**

Nonstandard Abbreviations and Acronyms

APC	activated protein C
CM	conditioned medium
EPCR	endothelial protein C receptor
HUVEC	human umbilical vein endothelial cell
PC	protein C
sEPCR	soluble endothelial protein C receptor
SNP	single-nucleotide polymorphism
VTE	venous thromboembolism

H3 is tagged by the minor allele of g.4600A>G (Ser219Gly; rs867186) and has been associated with the risk of venous[8–15,18,19] and arterial thrombosis,[19–23] but with contradictory results. The presence of H3 results in increased plasma sEPCR levels,[8–13,18,20,22,24] which is largely explained by a Ser219Gly substitution, which renders EPCR more susceptible to cleavage by metalloproteinases such as tumor necrosis factor-α converting enzyme/ADAM17.[7] Another mechanism that could link H3 to high plasma levels of sEPCR is its association with a truncated form of *PROCR* mRNA lacking the transmembrane and intracellular domains.[25] Recently, *PROCR* H3 has also been found to be associated with increased plasma levels of PC.[20,26]

Therefore, we aimed to verify the effects of H1 and H3 on VTE risk in a case–control study to investigate their effects on EPCR expression in human umbilical vein endothelial cells (HUVECs) and to identify the functional SNP that mediates the protection of H1 against VTE using luciferase constructs.

In the present study, we confirm previous reports on a protective effect of *PROCR* H1 against VTE[8,11–15] and show, for the first time, that it is probably mediated by the observed increase in functional membrane-bound EPCR in HUVEC carrying the H1 haplotype and the resulting enhanced rate of PC activation. As reported earlier, the H3H3 genotype was associated with increased sEPCR and increased VTE risk,[9] and we demonstrate, for the first time, that it is also associated with reduced functional membrane-bound EPCR levels. Using a modified luciferase reporter system, we could identify the g.2532T>C (rs2069948) SNP in intron 1 as a functional H1-specific SNP, although there are probably more.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Clinical Characteristics of the Subjects

The clinical characteristics of the study subjects are presented in Table 1. About 34% of the patients had spontaneous thrombosis, which is defined as thrombosis in the absence of known triggering factors (use of oral contraceptives, pregnancy, surgery, trauma, or immobilization). The prevalence of classical prothrombotic polymorphisms was similar to that described in other series.

PROCR Haplotypes and VTE Risk

The 702 patients and 518 control subjects were successfully genotyped for *PROCR* haplotypes H1 and H3. Table 2 shows the genotype distributions in patients and controls. To analyze the associations between genotypes and VTE, we performed logistic regression analyses. In univariate analysis, we confirmed that the presence of the H1H1 genotype significantly reduces the risk of VTE.[8,11–15] Adjustment for sex, age, and the presence of thrombophilic defects in a multivariate analysis did not significantly modify the odds ratio. We also confirmed that the presence of the H3H3 genotype is associated with an increased risk of VTE.[9]

APC, sEPCR, and PC Levels

Because variations in the *PROCR* gene contribute to circulating plasma PC,[20,26] APC,[8,11–13] and sEPCR[8–13,18,20,22,24] levels, we measured these levels in 462 patients with VTE (excluding those undergoing coumarin therapy) and 510 healthy individuals. As previously reported,[17] the levels of circulating APC were lower in patients with VTE (1.03±0.37 ng/mL) than in controls (1.25±0.40 ng/mL; $P<0.001$; Table 3). Both in patients and controls, APC levels increased with the number of H1 alleles ($P<0.001$), whereas they tended to decrease with increasing number of H3 alleles (Table 3).

Plasma sEPCR levels were similar in patients and controls (Table 3). When sEPCR levels were distributed according to genotypes, sEPCR levels strongly increased when the number of H3 alleles increased ($P<0.001$), confirming previous results.[8–13,18,20,22,24] There was no obvious effect of the H1 allele.

In agreement with previous reports,[20,26] both in patients and controls, antigen PC levels were significantly higher in carriers of the *PROCR* H3 allele than in noncarriers. Thus, PC levels were 101±17%, 103±19%, and 102±16% in controls carrying the HxHx, HxH1, and H1H1 genotypes, respectively, and 114±19% and 116±24% in controls carrying the HxH3 and H1H3 genotypes, respectively ($P<0.001$). In patients with VTE, PC levels were 100±21%, 103±20%, and 106±26% in carriers of the HxHx, HxH1, and H1H1 genotypes, respectively, and 122±17%, 110±18%, and 138±9% in carriers of the HxH3, H1H3, and H3H3 genotypes, respectively ($P<0.001$). These data indicate that the increased circulating APC levels observed in H1 carriers cannot be explained by an increase in PC levels in these individuals.

EPCR Protein and mRNA Levels in HUVECs

To further investigate whether the *PROCR* haplotypes H1 and H3 are functional and to identify the underlying mechanism, we used passage 2 HUVECs derived from 111 human umbilical cords from white newborns. Thirty-four HUVEC cultures were HxHx, 36 were HxH1, 27 H1H1, 5 H1H3, 8 HxH3, and 1 was H3H3. Figure 2 shows the concentration of EPCR in cell lysates and in conditioned medium (CM). EPCR levels in cell lysates increased ($P<0.001$), and sEPCR levels in the HUVEC-CM slightly decreased ($P=0.024$) with the number of H1 alleles. The total amount of EPCR measured in cell lysates and HUVEC-CM combined also increased with the number of H1 alleles (Figure 2C). In contrast, the presence of the H3 allele was associated with reduced levels of EPCR in cell lysates and, as expected, with increased levels in CM. This clearly establishes that the H3 allele leads not only to

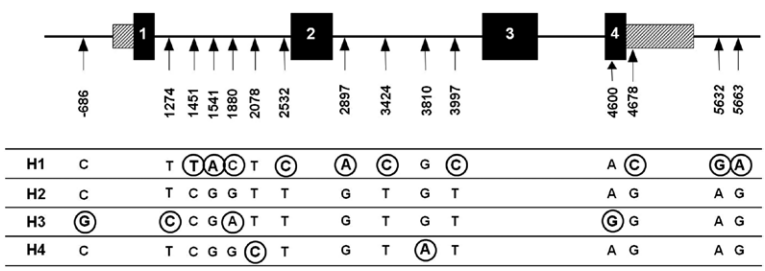

Figure 1. *PROCR* haplotypes and single-nucleotide polymorphisms (SNPs). Numbering is according to Simmonds and Lane.[4] Circled letters correspond to specific alleles for each haplotype. Italicized numbers indicate extrapolated numbering for these SNPs because the sequence described by Simmonds and Lane[4] does not reach these positions. The H1 haplotype contains 10 specific alleles, the 1451T (rs2069943), 1541A (rs2069944), 1880C (rs2069945), 2532C (rs2069948), 2897A (rs945960), 3424C (rs871480), 3997C (rs2069952), 4678C (rs9574), *5632G* (rs1415773), and *5663A* (rs1415774).

increased levels of sEPCR, but also to significantly reduced levels of EPCR on the cell membrane.

We also measured the *PROCR* full-length and truncated mRNA levels in 90 HUVEC samples for which mRNA isolated from passage 1 confluent cultures was available. Figure 3 shows the results obtained. There was a nonsignificant decrease in *PROCR* full-length mRNA levels with increasing number of H1 alleles. Similarly, there was a trend to lower *PROCR* full-length mRNA levels when the number of H3 alleles increased, although these differences were again not significant (Figure 3A). As previously reported,[25,27] a truncated mRNA form was detected in HUVECs, representing from 0.3% to 0.5% of the total *PROCR* mRNA in HUVECs not carrying H3 and from 1% to 2.3% in HUVECs carrying H3 (Figure 3B). This small amount of the truncated isoform does not explain the large increase in sEPCR levels observed in H3 carriers.

PC Activation on HUVECs

PC activation on HUVECs carrying different combinations of haplotypes was compared (Figure 4) to test the functionality of the membrane-bound EPCR. Because the thrombomodulin gene (*THBD*) g.1418C>T polymorphism has been associated with the rate of PC activation in cultured HUVECs,[28] we selected passage 2 HUVECs which were homozygous wild type for the *THBD* g.1418C>T SNP (1418CC). The rate of PC activation increased with the number of H1 alleles, whereas it decreased in the presence of H3. Preincubation of HUVECs with monoclonal antibody RCR-379 to block EPCR-dependent PC activation drastically reduced PC activation (Figure 4). In the same experiment, part of the HUVECs was used to measure the amount of EPCR in the cytosolic and cell membrane fractions. As seen in the top of Figure 4, HUVECs carrying the H1 haplotype have more membrane-bound EPCR than HUVECs not carrying the H1 haplotype, and HUVECs carrying the H3 haplotype have less membrane-bound EPCR than noncarriers. Cytosolic fractions did not contain detectable EPCR (<0.3 ng/mL).

Table 1. Characteristics of the Study Subjects

			Statistical Significance
	Patients	Controls	*P* Value
n	702	518	
Age, y, median	42 (33–53)	42 (32–52)	0.359
Age at first onset, y, median	39 (30–50)	…	
Male sex, n (%)	381 (54)	283 (55)	0.796
Pulmonary embolism, n (%)	175 (25)	…	
Recurrent thrombosis, n (%)	161 (23)	…	
Familial thrombosis, n (%)	147 (21)	…	
Spontaneous thrombosis, n (%)	239 (34)	…	
FV Leiden, n (%)			
–/–	604 (86)	497 (96)	
+/– and +/+	96 and 2 (14)	16 and 0 (3)	0.001
PT G20210A, n (%)			
GG	639 (91)	487 (94)	
GA and AA	63 and 4 (9)	31 and 0 (6)	0.001

FV indicates factor V; and PT, prothrombin.

PROCR H1 In Vitro Functional Studies

The H1 haplotype covers a much larger region (>250 kb) than the 6-kb *PROCR* gene itself; however, to date, no functional elements have been ascertained outside the region characterized by the SNPs (Figure 1). Therefore, to identify the H1 functional SNP(s), we focused initially on those SNPs encoded within *PROCR* itself. To study the functionality of the H1-specific SNPs in intron 1, intron 2, and the 3′ untranslated region (see Figure 1), we generated 3 sets of luciferase constructs in which we cloned *PROCR* intron 1, intron 2, or the 3′ untranslated region and downstream flanking region from H1 and H2, using the latter as the reference. After transfecting the human endothelial cell line EC-RF24 by electroporation, luciferase activity was measured. Constructs containing

Table 2. Distribution of *PROCR* Haplotypes in 702 Patients and 518 Control Subjects

			OR (95% CI)*	
Genotype	Cases, n (%)	Controls, n (%)	Crude	Adjusted*
HxHx†	150 (21.4)	100 (19.3)	1‡	1‡
H1Hx	293 (41.7)	192 (37.1)	1.02 (0.74–1.39)	1.02 (0.74–1.41)
H1H1	136 (19.4)	138 (26.6)	0.66 (0.46–0.93)	0.62 (0.43–0.89)
H1H3	53 (7.5)	48 (9.3)	0.75 (0.47–1.19)	0.70 (0.42–1.14)
HxH3	62 (8.8)	40 (7.7)	1.03 (0.64–1.66)	0.96 (0.59–1.56)
H3H3	8 (1.1)	0 (0)	12.07 (0.69–211.75)	…

CI indicates confidence interval; and OR, odds ratio.

*Adjusted for age, sex, and presence of thrombophilic defects (factor V Leiden, prothrombin 20210A, protein C deficiency, protein S deficiency, and antithrombin deficiency).

†x≠1 and 3.

‡Reference group.

Table 3. Levels of APC (mean±SD) and sEPCR (Median and 25th–75th Percentiles) in 462 Patients (Without Coumarin Therapy) and 510 Controls According to the *PROCR* Haplotypes

	APC, ng/mL					sEPCR, ng/mL				
Genotype	n	Patients	n	Controls	*P* Value	n	Patients	n	Controls	*P* Value
All	462	1.03±0.37	510	1.25±0.40	<0.001	462	101 (83–122)	510	100 (79–125)	0.216
HxHx	101	0.94±0.34	100	1.14±0.33	<0.001	101	86 (74–104)	100	88 (74–108)	0.278
HxH1	186	1.03±0.36	188	1.23±0.35	<0.001	186	98 (84–110)	188	91 (78–110)	0.463
H1H1	93	1.22±0.41	134	1.33±0.47	0.004	93	97 (81–111)	134	91 (75–112)	0.342
K–W test		<0.001		<0.001			0.335		0.523	
H1H3	34	0.98±0.32	48	1.36±0.44	<0.001	34	236 (207–300)	48	246 (196–282)	0.851
HxHx	101	0.94±0.34	100	1.14±0.33	<0.001	101	86 (74–104)	100	88 (74–108)	0.255
HxH3	41	0.97±0.29	40	1.20±0.31	<0.001	41	261 (212–295)	40	279 (211–326)	0.408
H3H3	7	0.64±0.25	0	…		7	491 (410–516)	0	…	
K–W test, *P* value		0.031		0.176			<0.001		<0.001	

x≠1 and 3. APC indicates activated protein C; K–W, Kruskal–Wallis; and sEPCR, soluble endothelial protein C receptor.

PROCR H1 intron 1 showed a significant reduction of 45% in luciferase activity compared with constructs containing H2 intron 1 (*P*=0.029). Constructs containing H1 intron 2 showed a 27% reduction in luciferase activity (*P*=0.071), and constructs containing the H1 3′ untranslated region and downstream flanking region showed a 14% increase in luciferase activity (*P*=0.383) compared with that of H2 (Figure 5).

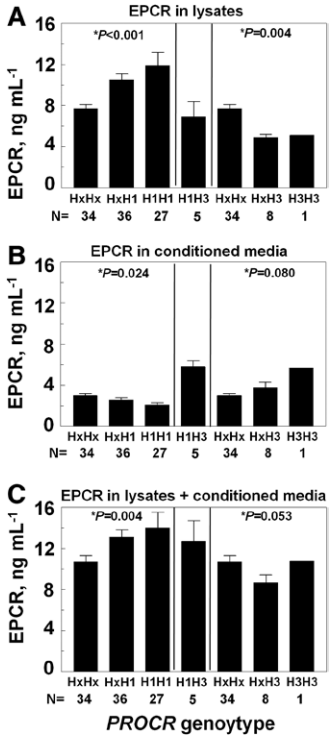

Figure 2. Endothelial protein C receptor (EPCR) levels in cell lysates and conditioned media from human umbilical vein endothelial cells (HUVECs), according to their *PROCR* haplotype. Passage 2 HUVECs were grown to 80% to 100% confluence and exposed for 3 hours to serum-free culture medium (50 μL/well). Thereafter, EPCR was measured in conditioned medium and membrane fraction as indicated in Materials and Methods in the online-only Data Supplement. **A**, Membrane fraction. **B**, Conditioned media. **C**, Membrane fraction plus conditioned media. Cytosolic fractions did not contain detectable EPCR (<0.3 ng/mL). Values are expressed as means±SEM. Hx≠H1 and H3. *Kruskal–Wallis test.

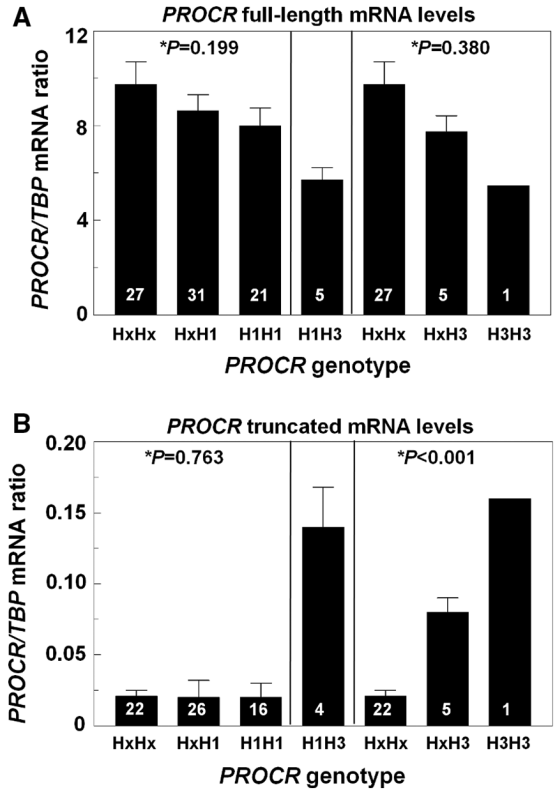

Figure 3. *PROCR* mRNA levels in human umbilical vein endothelial cells (HUVECs) according to *PROCR* haplotypes. Total RNA was isolated from passage 1 HUVEC confluent cultures, and real-time quantitative polymerase chain reaction was performed. Figures represent the relative level of *PROCR* mRNA compared with *TBP* mRNA (mean±SEM). Hx≠H1 and H3. **A**, *PROCR* full-length mRNA levels. **B**, *PROCR* truncated mRNA levels. *Kruskal–Wallis test.

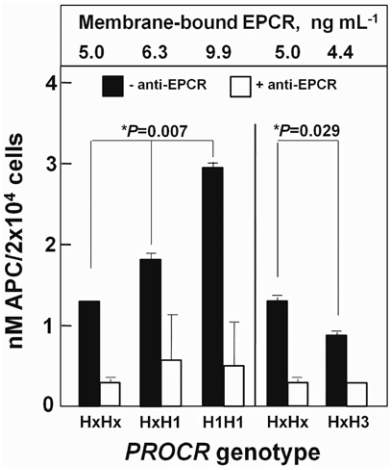

Figure 4. The rate of protein C (PC) activation increases with the number of *PROCR* H1 present. Passage 2 human umbilical vein endothelial cells (HUVECs) from 2 HxHx, 2 HxH1, 2 H1H1, and 2 HxH3 carriers were incubated with PC and thrombin at 37°C for 30 minutes as described in the Materials and Methods in the online-only Data Supplement. The activated PC (APC) generated/2×10⁴ cells is plotted against the corresponding genotype. The results represent the mean±SEM of 2 separate experiments performed in duplicate. Solid bars represent PC activation in the absence of anti–endothelial cell protein C receptor (EPCR) monoclonal antibody (mAb), RCR-379. Open bars represent PC activation in the presence of anti-EPCR mAb, RCR-379. In the absence of cells, ≈0.2 nmol/L of APC was generated. **Top**, Numbers represent the mean concentration of membrane-bound EPCR in the 2 HUVECs with the corresponding genotype. *Kruskal–Wallis test.

These results indicate that *PROCR* intron 1 might contain a functional SNP, and therefore, we generated, by site-directed mutagenesis, 4 sets of constructs in which we cloned each H1 SNP individually in the H2 background and again performed transfection experiments in EC-RF24 cells. As shown in Figure 6, only the construct containing the 2532C allele (rs2069948) showed a significant 34.4% reduction in luciferase activity compared with the construct containing H2 (*P*=0.003), a reduction that is similar to that obtained

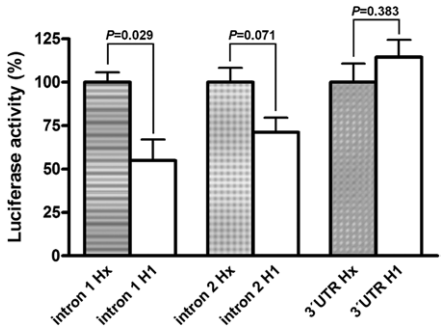

Figure 5. Luciferase activity of constructs containing *PROCR* intron 1, intron 2, or 3′ untranslated region (UTR) and downstream flanking region. Luciferase activity was measured 48 hours after transfection of EC-RF24 cells with constructs containing the *PROCR* H1 intron 1 or the H2 intron 1, constructs containing the H1 intron 2 or the H2 intron 2, and constructs containing the H1 or H2 3′ UTR and downstream flanking region. Data are expressed as mean±SEM of 3 to 5 experiments, in which ≥2 DNA preparations of each construct (wild type [wt] and mutant) were transfected in triplicate. Finally, the expression level of the mutant construct was calculated relative to the wt construct (H2), which was set at 100%.

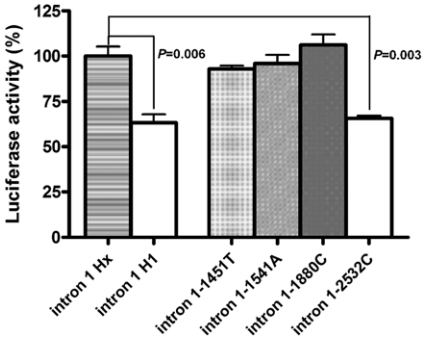

Figure 6. Luciferase activity of constructs containing each single-nucleotide polymorphism of *PROCR* intron 1 H1 mutated individually. Luciferase activity was measured 48 hours after transfection of EC-RF24 cells with constructs containing the *PROCR* H2 intron 1, the H1 intron 1, and the intron 1 with the 1451T allele (rs2069943), the 1541A allele (rs2069944), the 1880C allele (triallelic; rs2069945), and the 2532C allele (rs2069948). Data are expressed as mean±SEM of 3 to 5 experiments, in which ≥2 DNA preparations of each construct (wild type [wt] and mutant) were transfected in triplicate. Finally, the expression level of the mutant construct was calculated relative to the wt construct (H2), which was set at 100%.

when all 4 H1-specific SNPs are included compared with H2 (36.7%; *P*=0.006). Therefore, the g.2532T>C SNP seems to be the functional SNP responsible for the *PROCR* H1 effect observed in this in vitro model.

Discussion

In the present study, we confirmed that carriers of the H1H1 genotype in the *PROCR* gene have normal plasma sEPCR levels, higher levels of circulating APC, and a lower risk of VTE than those carrying other genotypes. We have shown for the first time that HUVECs carrying the H1 allele have significantly higher levels of membrane-bound EPCR and slightly lower sEPCR levels in HUVEC-CM than noncarriers. In agreement with this finding is the observation that the EPCR-dependent thrombin-mediated PC activation on intact HUVECs, as calculated from the experiments adding anti-EPCR (Figure 4), was higher in H1 carrying cells than in non-H1 carrying cells. Together, these findings indicate that the reduced risk of VTE of H1H1 carriers is associated with an increased concentration of functional EPCR on the endothelial cells. The latter might be responsible for the association of the H1 allele with increased plasma APC concentrations. In contrast, the *PROCR* full-length mRNA levels were similar in HUVECs from H1 carriers and non-H1 carriers, which suggests that the increase in functional membrane-bound EPCR associated with H1 is not caused by an increase in gene expression.

Several studies have investigated the association between *PROCR* haplotypes and venous and arterial thrombosis, with conflicting results. Some studies showed a reduced risk of VTE in carriers of the H1H1 genotype,[8,14,15] whereas others did not find such an effect.[9,10] An explanation for at least part of these discrepancies may lie in differences in study design and patient populations. Our present study is the largest case–control study performed with this aim.

We observed that the H3H3 genotype was associated with increased sEPCR levels and an increased risk of VTE, with

higher sEPCR levels in HUVEC-CM and, for the first time, with reduced functional membrane-bound EPCR levels. This reduction in functional membrane-bound EPCR seems not to be attributable to reduced gene transcription because the levels of *PROCR* full-length mRNA did not vary in the presence of H3. As expected, the levels of *PROCR* truncated mRNA significantly increased with the number of H3 alleles. It is however unlikely that this is the cause of the large increase in sEPCR levels observed in H3 carriers.[25]

The increased risk of VTE associated with the H3H3 genotype is in agreement with previous reports.[9,12,19] However, its mechanism is not fully understood. Our data suggest that it could be mediated by the increased EPCR shedding from the endothelial membrane, leading to significantly lower EPCR levels on the cell membrane and to a large increase in sEPCR levels. This effect of the H3 haplotype might be explained by the Ser219Gly substitution (rs867186), which would render EPCR more susceptible to cleavage by metalloproteinases such as ADAM17.[24] Qu et al[24] also showed that HUVECs carrying the H3 haplotype produced more sEPCR in CM than those carrying the H1 haplotype and that this resulted in reduced EPCR on the endothelial membranes. Unfortunately, they did not completely separate H1 from H3 in their calculations, making it difficult to compare their results with our present data.

We know that the H1 haplotype covers a much larger region (>250 kb) than the *PROCR* gene itself and that the functional variation(s) responsible for the observed associations might be located anywhere in this haplotype. To date, functional elements have not been described in the long region upstream of the *PROCR* gene that is also part of H1. Therefore, to identify SNPs responsible for the protective effect of H1, we restricted our in vitro analysis to the 10 H1-specific SNPs encoded within the *PROCR* gene. Using a modified luciferase reporter system, we identified the g.2532T>C SNP (rs2069948) in intron 1 as a plausible functional SNP in this part of the H1 haplotype. In our reporter system, this SNP is associated with reduced protein expression most likely by influencing splicing efficiency. The g.2532T>C SNP is located 16 bases from the intron 1–exon 2 splicing site in the branch point consensus sequence YTNAY, with 2532T/C at the position of the first pyrimidine (C or T) residue and the consensus A being used for the branch site. The 2532C allele is phylogenetically well conserved and is the ancestral allele, which suggests that this SNP might be functional. Interestingly, the g.2532T>C SNP reduced protein expression in our in vitro model system, which is opposite of what might be expected based on the observed association between H1 and EPCR expression in HUVECs and on the predictions of an in silico analysis using the Human Splicing Finder[29] (data not shown). On the contrary, it might be in agreement with the trend of reduced *PROCR* mRNA expression with increasing number of H1 alleles. Definite answers await further functional analysis of the H1-specific SNPs outside the *PROCR* gene itself.

In the present study, we have contributed to the further elucidation of the mechanism by which *PROCR* haplotypes 1 and 3 influence the risk of VTE. Our results show that the protective effect of H1 is associated with increased levels of functional membrane-bound EPCR, which will lead to an enhanced rate of PC activation. In contrast, the increased risk of VTE associated with H3 may be explained by the decrease in functional membrane-bound EPCR observed in HUVECs carrying the H3 haplotype.

Acknowledgments

We thank Marta Gurrea, Silvana Aniorte, Eugenia Romaguera, and Elisabet Rodríguez for collecting the umbilical cords, Úrsula Salinas and Josefa Llorens for technical assistance, and Haidy van der Putten for assistance in the in vitro functional experiments.

Sources of Funding

This work was supported by the Plan Nacional de I+D+I 2008 to 2011 (ISCIII PS09/00610 and PI12/00027), Fondo Europeo de Desarrollo Regional and Redes Red de Enfermedades Cardiovasculares (RD06/0014/0004), and Red de Investigación Cardiovascular (RD12/0042/0029), Consellería de Sanidad, Generalitat Valenciana (Prometeo 2011/027), and Instituto de Investigación Sanitaria La Fe, Spain. Pilar Medina is a Miguel Servet researcher (ISCIII CP09/00065).

Disclosures

None.

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Significance

Endothelial protein C receptor (EPCR) is an endothelial cell receptor that enhances the rate of protein C activation. It is encoded by *PROCR*. *PROCR* haplotypes have been associated with venous thromboembolism (VTE) risk, therefore we aimed to investigate the effects of *PROCR* H1 and H3 on EPCR expression and function. We have demonstrated that H1H1 individuals have increased plasma levels of activated protein C and reduced VTE risk. In human umbilical vein endothelial cells, we demonstrated that H1 is associated with increased levels of membrane-bound EPCR and an enhanced rate of protein C activation. In contrast, H3H3 carriers have increased plasma soluble EPCR levels and an increased VTE risk. Likewise, in cultured human umbilical vein endothelial cells, we demonstrated a decrease in the levels of functional membrane-bound EPCR, which might explain increased VTE risk associated with the H3H3 genotype. Finally, we identified the g.2132T>C single-nucleotide polymorphism in intron 1 as an intragenic H1-specific functional single-nucleotide polymorphism. With our investigation, we have contributed to further elucidation of the mechanism by which *PROCR* haplotypes influence the risk of VTE.

Material and methods

Patients and controls

The study included 702 unrelated Caucasian patients with VTE. Patients with an objectively confirmed episode of VTE who consecutively entered the anticoagulation clinic in our hospital for thrombophilic study between 1997 and 2012, were enrolled. Objective diagnoses of VTE and pulmonary embolism were made by clinical probability, D-dimer levels, compression ultrasonography, ventilation perfusion lung scan and, when necessary, phlebography or pulmonary angiography. Patients with known malignant disorders were excluded. The control group included 518 unrelated healthy subjects with no history of thromboembolic disease. Controls were randomly selected to match cases by age, gender and geographic distribution.

All subjects gave their informed consent to enter the study, which was approved by the Ethics Committee of our institution, and was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Blood collection

Blood was collected at least 6 months after the acute event. At the time of blood collection 240 patients were still on oral anticoagulants. For the thrombophilic study and the measurement of sEPCR, blood was collected in vacuum tubes containing 0.129 M trisodium citrate. For the measurement of circulating APC, blood was collected as previously reported[1,2]. Blood was centrifuged at 1500 x g for 30 min at 4 ºC. Plasma was frozen and stored at -72 ºC until testing. Tubes containing EDTA were used to collect blood for DNA studies.

Plasma sEPCR, protein C and APC

Levels of sEPCR in plasma were measured with the Asserachrom sEPCR ELISA kit (Diagnostica Stago, Asniéres-sur-Seine, France). The intra- and inter-assay coefficients of variation were less than 7%.

Plasma protein C[3] and circulating APC levels[1,2] were measured as previously reported.

Isolation of HUVECs

HUVECs from 111 umbilical cords were obtained by collagenase digestion and grown to confluence in T-75 flasks, as previously described[4].

The biochemical and cellular behaviors of HUVECs may be altered after several passages. Over 5-7 passages, cells gradually start to increase in size, to grow more slowly and to lose specific functions. Therefore, we used passage 1 HUVECs for DNA isolation and mRNA quantification, and passage 2 HUVECs for EPCR measurement and protein C activation experiments.

EPCR measurement in HUVECs

For measurement of EPCR in HUVEC conditioned medium (CM) and cell membrane and cytosolic fractions, cells were plated in 96-well culture plates, at a density of approximately 20 x 10^3 cells/well, and grown to reach 80-100% confluency in culture medium as previously described[4]. Cells were treated with 50 µL of lysis buffer pH 7.4 per well (10 mM Tris, 0.5% dithiothreitol, 10% glycerol, 1.5 mM EDTA), during 2 hours at 4ºC, with constant shaking. After centrifugation at 126,000 x g for 15 min at 4ºC, the supernatants (cytosolic fractions) were stored at -72ºC. The pellets were solubilized with 50 µL per well of 20 mM Tris-HCl, 125 mM NaCl, 1% Triton X-100, pH 7.4, vortexed for 5 min at 4ºC and centrifuged at 126,000 x g during 15 min at 4ºC. The supernatants (membrane fractions) were stored at -72ºC.

Protein C activation on HUVECs

Protein C activation on HUVECs was studied as described before[4]. Briefly, 100 nM protein C and 2 U/ml bovine thrombin (final concentrations) were added to confluent HUVECs in 96-well plates. After 30 min at 37 ºC, the reactions were stopped by addition of 50 µL of hirudin (50 U/ml) and 75 µL aliquots of the supernatants were transferred into 96-well microplates where the amidolytic activity of APC was measured by adding 15 µL of 8 mM S-2366. Under the conditions used in this study, <10% of the PC was activated during the incubation. All measurements were performed in duplicate. Where indicated, HUVECs were pretreated with 50 µg/ml of rat monoclonal anti-EPCR antibody RCR-379 (Abcam, Cambridge, UK) for 15 min before the addition of PC and thrombin. This antibody blocks the ability of EPCR to enhance protein C activation by thrombomodulin.

Genotyping of the PROCR H1 and H3 haplotypes

Genomic DNA was isolated from patients, controls and HUVECs using the Wizard Genomic DNA purification kit (Promega, Madison, WI), following the manufacturer's instructions.

PROCR H1 and H3 are tagged by the rs9574 and rs867186 SNPs, respectively, and these SNPs were genotyped as previously described[5,6].

mRNA isolation and real-time quantitative PCR analysis

Isolation of total RNA from HUVECs and synthesis of first strand cDNA was carried out as previously reported[4].

Quantification of the two *PROCR* mRNA transcripts (full-length and truncated mRNA) was performed following the previously reported protocol[7]. The results were normalized using the TATA-binding protein (TBP) transcript. Primer sequences are available on request. RT-qPCR was carried out using semi-automatic equipment (Light-Cycler Real-Time PCR Detection System, Roche, Mannheim, Germany). Each 15 µL reaction contained 2 µL of 1/10 diluted cDNA, 1.5 µL of 10X LightCycler® FastStart DNA Master Mix, 3 mM $MgCl_2$, 5 pmol μL^{-1} of the *PROCR* primer mix or 6 pmol μL^{-1} of the *TBP* primer mix. The amplification reaction for the *PROCR* cDNA was initially incubated at

95 ºC for 10 minutes, followed by 40 cycles of 5 seconds at 95 ºC, 10 seconds at 60 ºC, and 6 seconds at 72 ºC. The amplification reaction for the *TBP* cDNA was initially incubated at 95 ºC for 8 minutes, followed by 40 cycles of 15 seconds at 95 ºC, 5 seconds at 59 ºC, and 10 seconds at 72 ºC.

PROCR H1 in vitro functional study

To study the functionality of the *PROCR* H1-specific SNPs in intron 1, intron 2 and the 3´UTR with the downstream flanking sequence, we amplified these *PROCR* regions from homozygous carriers of H1 and H2 (intron 3 is identical in both haplotypes), using the *Pfu Ultra* Hotstart DNA Polymerase (Stratagene). H2 was used as the reference sequence, because it contains the common allele of all SNPs. The fragments were cloned into a modified pGL3-Basic vector (Promega). This pGL3-Basic vector was adapted by engineering SexAI and EcoRV restriction sites around the original position of the firefly luciferase intron 1, without affecting the encoded amino acids, to generate a system for testing intronic variants (van der Putten HH, Bertina RM, Vos HL, unpublished data). Introns 1 or 2 were cloned in the position of the first intron of the original firefly luciferase gene using hybrid primers that contained a modified luciferase sequence containing the relevant SexAI or EcoRV restriction sites followed by the ends of the relevant human *PROCR* introns. The DNA fragments with the 3´UTR and the downstream flanking region containing all sequences required for polyadenylation were cloned downstream from the luciferase cDNA (using XbaI and BamHI restriction sites), thereby replacing the SV40 late polyadenylation site normally present in pGL3-Basic. The CMV promoter was cloned upstream of the luciferase gene to drive its transcription. Primer sequences and cycling conditions are available upon request. Plasmid DNA was isolated using the PureYield Plasmid Maxiprep System (Promega).

The constructs were transiently transfected into the human endothelial cell line EC-RF24 (a kind gift from Dr. Hans Pannekoek[8]) by electroporation. In all cases, the pRL-CMV vector encoding the Renilla luciferase was co-transfected to correct for differences in transfection efficiency. Briefly, cells were trypsinized at 60-80% confluency and resuspended in RPMI 1640 with 10% fetal bovine serum. 550,000 cells were mixed with 2 µg of the construct of interest and 100 ng of pRL-CMV control vector DNA, and the mixture was electroporated at 220V and 1075 µF in a GenePulserII electroporator (Bio-Rad). The cell suspension was distributed in 3 wells of a 12-well plate containing complete M199 medium[8]. After 48 h culture, cells were lysed mechanically by constant shaking for 15 min at room temperature in 200 µl passive lysis buffer (Promega) followed by a freeze-thaw cycle at -72ºC. Subsequently, firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) in a GLOMAX 96 microplate Luminometer (Turner Biosystems). Activities were expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

The construct containing *PROCR* intron1 H1 seemed to contain a functional SNP, because of a difference in luciferase activity between the H1 and H2 constructs, therefore it was investigated in more detail. By using the Quikchange Site-Directed Mutagenesis Kit (Stratagene) we generated 4 sets of constructs each containing one of the 4 H1-specific SNPs in intron 1 in an H2-

background. Then we performed a new set of transfections as previously described.

Statistical analysis

The allelic distribution was analyzed with the HaploView program. Other statistical analyses were conducted using the SPSS for Windows release 11.5 statistical software (SPSS Inc., Chicago, IL). Data are reported as medians and interquartile ranges (25th - 75th percentiles) or means ± SEM. Allele frequencies were calculated by gene counting. The Chi-squared test was used to compare percentages. Parameter levels were compared with the Mann-Whitney U-test or the Kruskal-Wallis One ANOVA test. Correlations were assessed by the Spearman test. Logistic regression analysis was performed to identify the associations between genotypes and VTE risk. Multivariate analysis was performed using multiple logistic regression by including all the significant covariates in a single step. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from the logistic model. As none of the control individuals carried the H3H3 genotype, the OR for the *PROCR* H3-containing genotypes was calculated by assigning 0.5 to the number of controls with this genotype. Transfection results of each set of constructs were expressed as mean ± SEM of 3-5 experiments, in which 2 or more DNA preparations of each construct were transfected in triplicate. Finally, the expression level of the H1-derived constructs was calculated relative to that of the H2-derived construct, which was set as 100%. Luciferase activities of wt and mutant constructs were compared with an unpaired t-test using the GraphPad Prism® Software. Any differences with a two-tailed *P* value of <0.05 were considered statistically significant.

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Thrombosis Research 129 (2012) 459–464

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Haplotypes of the endothelial protein C receptor gene and Behçet's disease

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ARTICLE INFO

Article history:
Received 13 June 2011
Received in revised form 14 July 2011
Accepted 15 July 2011
Available online 17 August 2011

Keywords:
Behçet's disease
PROCR haplotypes
Activated protein C
Soluble EPCR
Venous thrombosis

ABSTRACT

Introduction: Behçet's disease is a vasculitis of unknown cause in which thrombosis occurs in about 25% of patients. Two haplotypes of the endothelial protein C receptor gene, H1 and H3, are associated with the risk of thrombosis. Thus, the objective of this study was to evaluate the influence of these haplotypes on the thrombosis risk in Behçet's disease.
Material and Methods: We evaluated the H1 and H3 haplotypes in 87 patients with Behçet's disease, 19 with and 68 without a history of thrombosis, and in 260 healthy individuals. We also measured protein C, activated protein C, and soluble endothelial protein C receptor levels in all individuals.
Results: The presence of the H1 haplotype seemed to protect Behçet's patients against thrombosis (odds ratio 0.21; 95% CI 0.1-0.8; $p=0.023$), whereas the frequency of the H3 haplotype was lower in patients than in control individuals (0.19; 0.1-0.5; $p=0.006$). Furthermore, the H1 haplotype was associated with increased levels of activated protein C, whereas the H3 haplotype was associated with the highest soluble endothelial protein C levels. Moreover, activated protein C levels were lower in patients with than in patients without posterior uveitis ($p<0.001$).
Conclusions: These findings indicate that the H1 haplotype protects Behçet's patients from thrombosis, likely via increased levels of activated protein C, whereas individuals carrying the H3 haplotype seem to be protected from the clinical manifestations associated with Behçet's disease, probably via increased soluble endothelial protein C levels.

Behçet's disease is a relatively rare autoimmune disease associated with damage to blood vessels throughout the body, particularly veins, but the exact cause of Behçet's disease remains unknown. This disease is characterized by recurrent oral and genital ulceration, in addition to skin lesions [1,2], involving cutaneous, articular, neurological intestinal, pulmonary, urogenital, and vascular manifestations [3,4]. Large vessels are affected by vasculitis of the vasa vasorum, and there is a prevalence of vascular involvement manifested by venous and arterial thrombosis in approximately 25% of patients [3,5,6]. Moreover, venous thromboembolism (VTE) is more common than arterial thrombosis, with deep vein thromboses being the most frequent diagnosis [7]. Endothelial dysfunction due to vascular inflammation is considered to be an important factor for developing thrombosis, although the endothelial injury itself cannot completely explain the hypercoagulable state of the disease, because other vasculitis syndromes do not increase the risk of thrombosis [8]. In fact, hypercoagulability and hypofibrinolysis also seem to be important factors in the development of thrombosis [4,9–12].

Most symptoms of Behçet's disease are caused by vasculitis, and include mouth sores, genital sores, uveitis, and skin problems. Inflammation inside of the eye (uveitis and retinitis) occurs in about half of Behçet's disease patients. Anterior uveitis (inflammation in the front of the eye) results in pain, blurry vision, light sensitivity, tearing, and/or redness of the eye. Posterior uveitis (inflammation in the back of the eye) may be somewhat more dangerous and even vision-threatening because it often causes fewer symptoms while damaging a crucial part of the eye—the retina [13,14].

It has been reported that, during endothelial injury, there is a generalized activation of the protein C pathway [15], which suggests that this pathway is intimately involved *in vivo* in the interaction of the coagulation and inflammatory systems [16]. This finding also highlights the interest in screening the protein C system in clinical situations in which the inflammatory process is involved in a variety

Abbreviations: VTE, venous thromboembolism; APC, activated protein; EPCR, endothelial protein C receptor; sEPCR, soluble EPCR; MI, myocardial infarction; *PROCR*, endothelial protein C receptor gene; SD, standard deviation; AT, antithrombin; PCI, protein C inhibitor; vWF, von Willebrand factor; ELISA, enzyme-linked immunosorbent assay; CRP, C-reactive protein; OR, odds ratio; CI, confidence interval.

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doi:10.1016/j.thromres.2011.07.032

of vasculitides. Furthermore, a reduced activated protein C (APC) level is an independent risk factor of both venous [17] and arterial thromboses [18] and has been associated with a high incidence of VTE in Behçet's disease [10]. Thrombin activation of protein C occurs on the endothelial cell surface following the formation of a quaternary complex comprised of thrombin, thrombomodulin, protein C, and endothelial protein C receptor (EPCR) [19,20]. EPCR seems to play an important role *in vivo*, as overexpression of EPCR alters the hemostatic balance and protects mice from endotoxin, by increasing the levels of circulating APC [21], and blockade of EPCR by specific antibodies accelerates thrombus development in vivo [22].

Two haplotypes of the *PROCR* gene, H3 and H1, may modify the risk of developing thrombosis [23]. *PROCR* H3, which is tagged by the single nucleotide polymorphism (SNP) 4600A/G (rs867186), seems to be functional, as it is associated with both increased plasma levels of soluble EPCR (sEPCR) [24–28] and increased EPCR shedding *in vitro* [27,29], but its association with the risk of VTE is unclear. Two previous studies failed to find an association between the H3 haplotype and the risk of VTE in Spanish and Dutch subjects [25,26], but this haplotype was shown to be overrepresented in both male patients with VTE in a French study [24], and in carriers of the prothrombin 20210A allele in a Spanish study [28]. Furthermore, in a pan-European case-control study of myocardial infarction (MI), Ireland *et al.* [27] reported that, without the challenge of diabetes or metabolic syndrome, individuals carrying one H3 haplotype were protected from MI. In addition, Medina *et al.* [30] showed that carriers of the H3 haplotype had a reduced risk of premature MI, in part due to its association with high sEPCR levels. *PROCR* H1 haplotype, which is tagged by the 4678 G/C SNP (rs9574), is associated with both increased levels of circulating APC and a reduced risk of venous [25,28,31] and arterial thromboses [30].

The aim of the present study was to investigate whether these two functional *PROCR* haplotypes are associated with the risk of thrombosis in patients with Behçet's disease.

Material and Methods

Patients

In this study, we examined 87 patients with Behçet's disease (48 male, 39 female), of whom 39 were partially assessed in a previous study [10]. The patients were recruited via the files of the Internal Medicine Service of our hospital. The mean (± standard deviation, SD) patient age at diagnosis was 33 ± 12 years (range, 2 − 60 years). The mean age at study inclusion was 45 ± 12 years (range, 19 − 74 years). All patients fulfilled the criteria for diagnosis of Behçet's disease according to the International Study Group for Behçet's disease [32]. Only 3 patients with Behçet's disease presented severe ocular affectation at the time of sampling, which indicates that they were in an active phase of the disease. In all other patients, the disease was inactive or had minimum activity (mild aphthosis and/or arthralgias). Uveitis was diagnosed by ophthalmic examination and fluorescein angiography.

Nineteen patients had suffered a VTE and were referred to our service for thrombophilic study; the thromboses involved the lower limbs ($n = 14$), iliac-cava vein plus pulmonary embolism ($n = 1$), cerebral sinus ($n = 1$), right intracardiac area ($n = 1$), upper limbs ($n = 1$), and pulmonary embolism plus ischemic stroke ($n = 1$). Four patients had suffered repeated episodes of superficial phlebitis. Furthermore, four patients had experienced more than one VTE episode and were on long-term oral anticoagulant therapy with acenocoumarol. According to the protocol approved by the Ethics Committee of our hospital, the thrombophilic study of these four patients who had experienced multiple VTE events was performed as follows: 20 days before sampling, acenocoumarol was replaced by low-molecular weight heparin, and heparin was not administered on the day before sampling. None of these patients had antiphospholipid antibodies, malignancy, renal or hepatic dysfunction, or lupus anticoagulant. Moreover, none of these patients were receiving oral anticoagulation therapy at the time of sampling. Sampling took place at least 6 months after the thrombotic event.

The thrombophilic study, which was performed on all enrolled patients, evaluated the following parameters: antithrombin (AT), protein C, protein S, APC resistance, factor V Leiden, and the prothrombin G20210A mutation.

Objective thrombosis diagnosis procedures were performed on all patients to confirm the diagnosis of either VTE (contrast venography or ultrasonography) or pulmonary embolism (ventilation-perfusion lung scanning or pulmonary angiography). Furthermore, intracardiac thrombosis was assessed by transoesophageal echocardiography. Cerebral venous thrombosis and ischemic stroke were diagnosed by computed tomography, magnetic resonance imaging, and venography.

At the time of blood collection, 16 patients were taking corticosteroids, 12 were under colchicine treatment, and 10 were taking both drugs. Nineteen patients were taking cyclosporine, either alone ($n = 6$) or in combination with corticosteroids ($n = 8$) or colchicine ($n = 5$), and 30 patients were not receiving specific treatment.

Controls

While patients were being recruited, a control group of 260 age- and sex-matched healthy individuals from the same geographical area as the patients was studied. In particular, these individuals were partners of the patients (N = 60), laboratory and medical personnel and friends or partners of them (N = 100), and persons who visited our hospital for medical checkups (N = 100). All controls were apparently healthy and none had a personal or familial history of thrombotic disease. The absence of thrombotic events and a family history of thrombosis were verified by means of a validated questionnaire [33]. The thrombophilic study was carried out in the control group individuals using the same protocol as for the Behçet's disease patients. All patients and control individuals provided informed consent, and the study was approved by our local Ethics Committee.

Laboratory tests

Plasma APC levels were measured using a previously reported method [34] with slight modifications [17]. Briefly, 4.5 ml of blood was drawn into two tubes containing 0.5 ml of 0.129 mol/l trisodium citrate. Immediately, 46 μl of 1000 U/ml heparin was added to one of the two tubes, and the mixture was incubated at 37 °C for 30 min to force all plasma APC to form complexes with its major plasma inhibitor, protein C inhibitor (PCI) [35]. The second blood tube was mixed with 46 μl of a mixture of 0.58 mol/l benzamidine HCl and 0.5 mmol/l PPACK to inhibit circulating APC. The detection limit of the APC assay was 0.1 ng/ml. The inter- and intra-assay variations were less than 6% and 11%, respectively. Furthermore, repeated assays revealed non-significant intra-individual variations of APC levels [17].

For the other parameters, blood samples were collected into vacuum tubes containing 1/10 vol. of 3.2% trisodium citrate as an anticoagulant. Samples were centrifuged at 1500 × *g* for 30 min to obtain platelet-poor plasma samples, which were stored in aliquots at −70 °C until they were tested.

Levels of sEPCR were measured using a commercially available ELISA (Asserachrom sEPCR, Diagnostica Stago, Asnieres, France), according to the manufacturer's instructions. The inter- and intra-assay variations were both less than 9%. The functional levels of fibrinogen and factor VIII were determined using a high sensitivity standard reagent from Instrument Laboratory (IL, Milano, Italy). The inter- and intra-assay variations were less than 5% and 10%, respectively. Quantification of von Willebrand factor (vWF) was performed using an immunoturbidimetric assay [36], with an IL Test vWF:Ag Kit (IL). The IL Calibrator Plasma for

vWF:Ag was calibrated against the 4th International Standard FVIII/VWF (NIBSC97/586). The inter- and intra-assay variations were both less than 4%. The AT activity was assayed for its anti-activated factor X activity in the presence of heparin using the IL test Antithrombin Kit (IL). The inter- and intra-assay variations were both less than 4%. Protein S activity was determined as the degree of prolongation of the prothrombin time in the presence of bovine thromboplastin, calcium ions and APC, using the IL test (IL). The inter- and intra-assay variations were both less than 7%. The inter- and intra-assay variations were both less than 5%. Protein C antigen [37] and APC:PCI complex levels [38] were measured using ELISAs according the protocol described earlier. The inter- and intra-assay variations were less than 6% and 9%, respectively. C-reactive protein (CRP) was measured by a high-sensitivity nephelometric assay (Behring Nephelometer, Behringwerke AG). The inter- and intra-assay variations were both less than 3%.

Genomic DNA was extracted from peripheral blood using standard methods. Factor V Leiden mutation was detected by polymerase chain reaction amplification and restriction analysis of a fragment of the Factor V gene, according to the method described by Gandrille *et al.* [39]. The prothrombin gene variant *G20210A* was detected using a previously described technique [40]. *PROCR* H1 and H3 are tagged by the rs9574 and rs867186 polymorphisms, respectively, and these polymorphisms were genotyped as indicated in previous studies [25,42].

Statistical analysis

Statistical analyses were conducted using SPSS software for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). The distributions of the variables were assessed with Kolmogorov − Smirnov tests. Continuous variables are reported either as means and standard deviations (for normally distributed data), or as medians and 10th and 90th percentiles (for non-normally distributed data). Patient and control groups were compared with the Student's *t*-test for independent samples, or with the Mann − Whitney rank sum *U*-test for skewed distributions. The chi-square test (with Yate's correction) was used to compare the sex ratios, percentage of smokers, and the prevalence of thrombophilic mutations between the patients and controls. Correlations were evaluated with Pearson's linear regression or with the Spearman rank test. Logistic regression analysis was used to identify the links between the *PROCR* haplotypes and Behçet's disease. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated from the logistic model. Two-tailed *P*-values of 0.05 or less were considered statistically significant.

Results

The clinical characteristics of study groups are given in Table 1. We observed no significant differences between the patient and control groups in terms of age, sex distribution, and the percentage of smokers. Furthermore, we found no individuals in the patients group with protein S or AT deficiencies. However, one patient and two control subjects had protein C deficiency. Among the patients with Behçet's disease, three were heterozygous carriers of the factor V Leiden mutation, three carried the *prothrombin G20210A* variant, and one patient was heterozygous for both of these mutations. Two patients with the *prothrombin G20210A* variant and one with a combination of factor V Leiden and *prothrombin G20210A* variant had a history of VTE. In the control group, six individuals were heterozygous for the factor V Leiden mutation ($P = 0.277$) and 10 for the *prothrombin G20210A* mutation ($P = 0.756$).

Given that the control group was recruited from three heterogeneous sources, we investigated for a potential bias. No differences were found ($P > 0.4$) in the distribution of *PROCR* haplotypes or in the levels of coagulation and inflammation parameters between the three control subgroups.

Table 1
Clinical characteristics of the subjects examined in this study.

Parameter	Behçet's patients (n = 87)	Controls (n = 260)	*P*
Age (years, mean ± SD)	45 ± 12	45 ± 11	0.750
Male (%)	55.2	54.6	0.928
Smokers (%)	32.6	38.0	**0.389**
Mouth ulcers (%)	100		
Genital ulcers (%)	75		
Arthralgia (%)	65		
Fever (%)	53		
Ocular involvement (%)	40*		
Cutaneous involvement (%)	44†		
Thrombotic events (%)	22		
Neurological events (%)	29‡		
Gastrointestinal involvement (%)	12		

*21 patients with posterior uveitis, 17 with anterior uveitis, and 2 with both posterior and anterior uveitis.
†20 patients with pseudofolliculitis, 10 with erythema nodosum, 3 with vasculitis, and 5 with other diagnoses.
‡Central nervous system (23 patients) and peripheral nervous system (2 patients).

PROCR haplotypes and Behçet's disease

The allelic and genotypic frequencies of the rs9574 and rs867186 in *PROCR* are shown in Table 2. The presence of *PROCR* H3 was lower in patients than in controls (OR 0.29; 95% CI 0.12 − 0.70; $P = 0.006$). Furthermore, adjusting for age, sex, and the presence of thrombophilic defects did not significantly modify this association (OR 0.19; 95% CI 0.07 − 0.55; $P = 0.002$). After excluding the 19 patients with a history of VTE, these OR did not significantly change (OR 0.31 and 0.183, respectively). In contrast, the presence of the *PROCR* H1 was not significantly different in patients and controls.

PROCR haplotypes and risk of thrombosis

Since *PROCR* H1 and H3 have been associated with the risk of VTE, we evaluated the association of these *PROCR* haplotypes with the risk of VTE in Behçet's disease patients in this study (Table 3). The presence of *PROCR* H1 tended to be greater in the subgroup of patients without thrombosis (84%) than in the subgroup of patients with a history of thrombosis (63%) ($P = 0.056$). Logistic regression analyses showed that, after adjustment for age, sex, and the presence of thrombophilic defects (protein C, protein S and antithrombin deficiencies, factor V Leiden and prothrombin 20210A), the presence of *PROCR* H1 reduced the risk of thrombosis in Behçet's disease patients (OR 0.21; $P = 0.023$). In contrast, the presence of *PROCR* H3 was not associated with the risk of thrombosis (OR 1.59; $P = 0.716$).

Table 2
Association of the *PROCR* H1 and H3 haplotypes with Behçet's disease.

Polimorphism	Patients n (%)	Controls n (%)	Odds Ratio (95% CI)†	
			Crude	Adjusted$
rs9574				
Presence of H1	69 (79.3)	188 (72.3)	1.47 (0.82 − 2.64)	1.50 (0.81 − 2.61)
HxHx genotype	18 (20.7)	71 (27.3)	1*	1*
H1Hx genotype	47 (54.0)	129 (49.6)	1.44 (0.78 − 2.66)	1.41 (0.76 − 2.63)
H1H1 genotype	22 (25.3)	60 (23.1)	1.45 (0.71 − 2.95)	1.00 (0.97 − 1.04)
rs867186				
Presence of H3	6 (6.9)	53 (20.4)	0.29 (0.12 − 0.70)	0.19 (0.07 − 0.55)
HyHy genotype	81 (93.1)	207 (79.6)	1*	1*
H3Hy genotype	6 (6.9)	53 (20.4)	0.29 (0.12 − 0.70)	0.19 (0.07 − 0.55)

$x \neq 1$; $y \neq 3$
†CI, confidence interval.
*Reference group.
$Odds ratio adjusted for age, sex, and the presence of major thrombophilic risk factors.

Table 3
Allelic and genotypic frequencies of the SNPs in the *PROCR* gene in Behçet'sdisease patients with ($n=19$) and without ($n=68$) venous thromboembolism (VTE).

Polymorphism	Patients with VTE n (%)	Patients without VTE n (%)	$Odds Ratio (95% CI)
rs9574			
Presence of H1	12 (63)	57 (84)	0.21 (0.06 − 0.81) ($P=0.023$)
HxHx genotype	7 (37)	11 (16)	1.0*
H1Hx genotype	8 (42)	39 (57)	0.42 (0.11 − 1.65) ($P=0.248$)
H1H1 genotype	4 (21)	18 (26)	0.12 (0.01 − 1.25) ($P=0.077$)
rs867186			
Presence of H3	1 (5)	5 (7)	1.59 (0.13 − 19.03) ($P=0.716$)
HyHy genotype	18 (95)	63 (93)	1.0*
H3Hy genotype	1 (5)	5 (7)	1.59 (0.13 − 19.03) ($P=0.716$)

$x \neq 1$; $y \neq 3$
*Reference group.
$Odds ratio adjusted for age, sex, and the presence of major thrombophilic risk factors; CI, confidence interval.

APC and sEPCR levels, Behçet's disease, and VTE risk

Similar to results from a previous report [10], we found that the mean level of APC was significantly lower in the 87 patients with Behçet's disease than in the 260 control individuals ($P<0.001$), whereas the levels of fibrinogen, factor VIII, vWF, and CRP were significantly higher in the patients than in the control individuals (Table 4). Furthermore, the mean sEPCR level was significantly lower in the patient group than in the control group ($P<0.001$). (Table 4). These results did not significantly change when the 19 patients with a history of VTE were excluded from the analyses (data not shown).

Stratification of sEPCR into quartiles according to the distribution of the control group showed that, compared to the first quartile, the OR for the fourth quartile was 0.15 (95% CI 0.05 − 0.45). Adjustment for age, sex, and the presence of thrombophilic defects did not significantly modify the protection of sEPCR against Behçet's disease (OR 0.10; 95% CI 0.02 − 0.34; $P=0.001$)

Since high sEPCR levels are strongly associated with *PROCR* H3 (rs867186), we performed a bivariate analysis including both the fourth quartile of sEPCR and the presence of *PROC*R H3 in the model. The adjusted ORs were determined to be 0.10 (95% CI 0.01 − 0.46; $P=0.005$) and 0.92 (95% CI 0.14 − 5.9; $P=0.929$), respectively.

Given that *PROCR* H1 and H3 have been associated with increased plasma levels of APC [25,28,30] and sEPCR [24–28,30], respectively, we compared these parameters in patients with and without a history of thrombosis according to these haplotypes. We found that the APC levels increased with the number of H1 alleles present, both in the control individuals (0 alleles = 1.12 ± 0.25 ng/ml; 1 allele = 1.28 ± 0.37 ng/ml; 2 alleles = 1.40 ± 0.58 ng/ml; $P<0.001$) and in the Behçet's disease patients (0 alleles = 0.71 ± 0.18 ng/ml; 1 allele = 0.75 ± 0.16 ng/ml; 2 alleles = 0.92 ± 0.20 ng/ml; $P=0.010$). Furthermore, the level of sEPCR was significantly higher in carriers of *PROCR* H3 than in individuals without this haplotype, both in the control group (H3 carriers = 215 ng/ml, 95% CI 93 − 384; H3 non-carriers = 100 ng/ml, 95% CI 68 − 200; $P<0.001$) and in the patient group (H3 carriers = 178 ng/ml, 95% CI 156–212; H3 non-carriers = 90 ng/ml 95%, CI 66 − 119; $P<0.001$).

As reported previously [10], among the patients with Behçet's disease, the APC levels were lower in those with a history of thrombosis (0.68 ± 0.17 ng/ml) than in those without a VTE history (0.80 ± 0.18 ng/ml) ($P=0.026$), whereas no differences were found in the sEPCR levels between these two groups of patients ($P=0.702$).

APC and sEPCR, and uveitis

In general, patients with a history of ocular involvement ($n=35$) had lower APC levels than those without ocular involvement ($n=52$) (0.73 ± 0.18 and 0.85 ± 0.14 ng/ml, respectively; $P=0.001$). This difference was due to patients with posterior uveitis ($n=23$), who had significantly reduced APC levels (0.68 ± 0.16) compared with patients without posterior uveitis ($n=64$) (0.84 ± 0.14 ng/ml) ($P<0.001$), whereas patients with and without anterior uveitis had similar APC levels (0.77 ± 0.16 and 0.81 ± 0.16 ng/ml, respectively; $P=0.339$).

In contrast, no significant differences were observed in sEPCR between patients with and without ocular involvement ($P=0,145$), or between patients with and without posterior uveitis ($P=0,205$).

Inflammation markers

As reported earlier [10], the mean levels of α_1AT, fibrinogen, factor VIII, vWF and CRP were significantly higher in patients than in controls (Table 4). To assess the influence of the *PROCR* H3 haplotype on the levels of inflammation markers, we distributed these levels according to the presence or absence of this haplotype. Table 5 shows the results obtained. As previously reported (24–28), the level of sEPCR was higher in carriers of the *PROCR* H3 haplotype than in non-carriers. Among controls, carriers of the H3 haplotype also showed higher levels of fibrinogen, factor VIII, vWF and CRP than non-carriers ($P<0.034$). Among patients, carriers of the H3 haplotype tended to have higher levels of inflammatory markers, but the differences did not reach statistical significance.

PROCR H3 haplotype and protein C

Because it has been reported that the PROCR H3 haplotype is associated with increased protein C levels [42–44], we analyzed this association in our study. In the control group, the 53 carriers of the H3 haplotype had significantly higher protein C levels (117% ± 21%) than

Table 4
Coagulation and inflammation parameters in the patients and control individuals examined in this study.

Parameter	All patients (A) (n = 87)	Patients without VTE (B) (n = 68)	Controls (C) (n = 260)	*P*	
				A-C	B-C
Activated protein C (APC) (ng/ml)	0.76 ± 0.18	0.82 ± 0.16	1.21 ± 0.35	<0.001	<0.001
Soluble EPCR (ng/ml)	93 (68 − 123)	93 (66 − 122)	106 (69 − 270)	<0.001	<0.001
Protein C (U/ml)	1.16 ± 24	1.15 ± 18	1.06 ± 18	0.001	0.008
Antithrombin (U/ml)	1.03 ± 11	1.04 ± 12	1.01 ± 12	0.198	0.078
Fibrinogen (g/l)	3.28 ± 87	3.21 ± 86	2.73 ± 61	<0.001	0.007
Factor VIII (U/dl)	127 ± 48	125 ± 44	97 ± 25	<0.001	<0.001
Von Willebrand factor (U/dl)	128 ± 61	126 ± 54	96 ± 34	<0.001	<0.001
C-reactive protein (mg/l)	2.15 (0.43 − 16.37)	2.23 (0.37 − 15.90)	1.16 (0.37 − 3.34)	<0.001	<0.001

Data are expressed as mean ± SD, except for C-reactive protein, which is expressed as median (10th − 90th percentiles).

Table 5
Inflammatory markers in the patients and control according to the presence of *PROCR* H3.

Parameter	Patients			Controls		
	H3 carriers (n = 6)	H3 non-carriers (n = 81)	*P*	H3 carriers (n = 53)	H3 non-carriers (n = 207)	*P*
Fibrinogen (g/l)	2.68 ± 0.72	3.32 ± 0.87	0.082	2.53 ± 0.63	2.78 ± 0.60	0.008
Factor VIII (U/dl)	101 ± 30	129 ± 47	0.069	92 ± 23	100 ± 25	0.033
Von Willebrand factor (U/dl)	102 ± 28	130 ± 57	0.054	89 ± 20	97 ± 30	0.025
C-reactive protein (mg/l)	1.81 (1.21-13.04)	2.31 (0.44-18.50)	0.708	1.03 (0.22-2.21)	1.22 (0.44-2.83)	0.031

Data are expressed as mean ± SD, except for C-reactive protein, which is expressed as median (10th − 90th percentiles).

the 207 non-carriers (104% ± 17%). In patients, the 6 carriers of the H3 haplotype also showed increased protein C levels (129% ± 10%) compared to the levels found in the 81 non-carriers (115% ± 24%), although the difference was not significant ($P = 0.149$), probably due to the low number of patients with the H3 haplotype.

Influence of treatment on the levels of coagulation and inflammation parameters

Inflammation markers were, in general, lower in patients who were not under treatment for Behçet's disease, but the differences were not statistically significant (Table 5). Although APC levels in the subset of patients treated with immunosuppressors were lower than in the other two subgroups, as reported earlier [10], APC levels in the three subgroups of patients were lower than in the control group ($p < 0.001$ in all cases). The levels of sEPCR were similar in the three subgroups of patients, and were lower than in the control group.

Discussion

Our data indicate that *PROCR* H1 reduces the risk of VTE in Behçet's disease patients, via increased levels of circulating APC. Also of note, increased APC levels apparently protect patients from developing posterior uveitis. Our findings also reveal that carriers of *PROCR* H3 are protected against the clinical manifestations associated with Behçet's disease, and that this protection is likely due to the increased levels of sEPCR that are associated with this haplotype.

One previous study showed that the mean levels of sEPCR were significantly higher in patients with Behçet's disease than in control individuals [45], which is in conflict with our results. This difference may be due to different *PROCR* H3 frequencies in the two study populations. Although the authors of the previous study did not analyze *PROCR* haplotypes, their data on sEPCR levels suggest similar H3 frequencies in patients and controls, whereas we found that this frequency was lower in patients than in the control individuals. However, the aforementioned authors found no differences in the sEPCR levels in patients with and without ocular involvement, which is in agreement with our results.

In addition, we found that patients with posterior uveitis had significantly lower APC level than those without posterior uveitis, whereas no significant differences were found when patients with and without ocular involvement were compared. Theses data suggest that APC may protect patients with Behçet's disease from posterior uveitis. Although the mechanism for this protection may be related with the antiinflammatory functions attributed to APC, further studies are needed to confirm this hypothesis.

The mechanism by which *PROCR* H3 protects individuals from clinical manifestations associated with Behçet's disease is uncertain. The fact that this protective effect partly disappears after adjusting by sEPCR levels might indicate that the effect is via increased sEPCR levels. Behçet's disease is characterized by vascular inflammation. Several *in vivo* anti-inflammatory effects of APC require cell-bound EPCR [46]. However, whether sEPCR is able to preserve some of these activities remains to be elucidated, although it has been reported that, *in vitro*, sEPCR may bind to activated neutrophils via proteinase-3 and Mac-1 (CD11b/CD18) integrin, and may regulate vasculitis in Wegener's granulomatosis and related vasculitides [47]. In agreement with this hypothesis, it has been reported that carriers of the H3 haplotype have a reduced risk of premature MI [27,30], via increased sEPCR levels [30]. Furthermore, our results show that the level of several inflammation parameters including fibrinogen, factor VIII, vWF and CRP is lower in *PROCR* H3 carriers than in non-carriers (Table 5), which supports the protective role of sEPCR. However, due to the design of the case-control study, we cannot exclude that the low sEPCR levels seen in Behçet's disease patients reflect endothelial injury and decrease as a result of the disease.

The mechanism by which *PROCR* H1 reduces the risk of VTE in patients with Behçet's disease may be similar to that suggested for patients with venous and arterial thrombosis, which involves increased circulating APC levels in carriers of the *PROCR* H1 [25,28,30]. However, further studies are necessary to clarify this issue.

A limitation of our study is the low number of patients with VTE, which reduces the strength of our conclusions. Nevertheless, the finding that *PROCR* H1 reduces the risk of VTE has been reported in other studies with larger series of patients [25,28,31,41].

In conclusion, *PROCR* H1 protects Behçet's patients from thrombosis, via increased APC levels, whereas individuals carrying *PROCR* H3 are protected from the clinical manifestations associated with Behçet's disease, likely due to the association of this haplotype with increased sEPCR levels.

Conflict of interest statement

The authors reported no potential conflicts of interest.

Acknowledgments

We thank Úrsula Salinas for excellent technical assistance. This work was supported by grants from the PN de I + D + I 2008–20011, ISCIII, and FEDER (PS09/00610 to FE, Red RECAVA RD06/0014/0004 to FE, and RD06/0014/0016 to JF), the Consellería de Educación Generalitat Valenciana (Prometeo/2011/027 to FE), and the Fundación para la Investigación del Hospital Universitario La Fe (2007–0185 to FE), Spain. Pilar Medina is a Miguel Servet Researcher (ISCIII CP09/00065) and Silvia Navarro is a RECAVA Researcher (ISCIII RD06/0014/0004).

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Thrombosis Research 128 (2011) 410–416

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Review Article

The endothelial cell protein C receptor: Its role in thrombosis

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ARTICLE INFO

Article history:
Received 13 May 2011
Received in revised form 14 July 2011
Accepted 1 August 2011
Available online 7 September 2011

Keywords:
activated protein C
endothelial protein C receptor
factor VII
venous thrombosis
myocardial infarction

ABSTRACT

The protein C anticoagulant pathway plays a crucial role as a regulator of the blood clotting cascade. Protein C is activated on the vascular endothelial cell membrane by the thrombin-thrombomodulin complex. The endothelial protein C receptor binds protein C and further enhances protein C activation. Once formed, activated protein C down-regulates thrombin formation by inactivating factors Va and VIIIa and exerts cytoprotective effects through endothelial protein C receptor binding. An adequate generation of activated protein C depends on the precise assembly, on the surface of the endothelial cells, of thrombin, thrombomodulin, protein C, and endothelial protein C receptor. Therefore, any change in the efficiency of this assembly may cause a reduction or increase in activated protein C generation and modulate the risk of thrombosis. This review highlights the role of the endothelial protein C receptor in disease and discusses the association of its mutations with the risk of thrombosis.

Contents

Introduction

The protein C (PC) anticoagulant pathway plays a crucial role in the regulation of fibrin formation via proteolytic inactivation of the procoagulant cofactors Factor (F) Va and FVIIIa [1–3]. PC is a vitamin K-dependent plasma glycoprotein that circulates in plasma as an inactive zymogen, which is activated to activated PC (APC) [4] on the surface of endothelial cells by the thrombin-thrombomodulin (TM) complex. Another endothelial cell-specific protein, which is involved in the PC anticoagulant pathway, is the endothelial cell PC receptor (EPCR). EPCR binds PC to on the endothelial cell surface. This binding

Abbreviations: PC, protein C; F, factor; APC, activated protein C; TM, thrombomodulin; EPCR, endothelial protein C receptor; TNF-α, tumor necrosis factor-α; *PROCR*, endothelial protein C receptor gene; aa, amino acid; UTR, untranslated region; sEPCR, soluble EPCR; TF, tissue factor; PAR-1, protease-activated receptor-1; MAPK, mitogen activated protein kinase; VTE, venous thromboembolism; SNPs, single nucleotide polymorphisms; T, thrombin; PS, protein S.

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doi:10.1016/j.thromres.2011.08.001

enhances the rate of PC activation by the thrombin-TM complex by 5- to 20-fold [5] by decreasing the K_m of PC for its activation by the thrombin-TM complex. The relevance of the anticoagulant role of EPCR in vivo has been well documented: EPCR blockade in baboons notably reduced the amount of APC generated upon thrombin administration [6], and an anti-EPCR monoclonal antibody accelerated thrombus development in a murine model of thrombosis [7].

TM is uniformly distributed on all endothelial cells, which results in relatively low TM concentrations in large vessels. This would result in inefficient activation of PC in these vessels [8]. This effect is counterbalanced by EPCR, which is highly expressed on the endothelium of large vessels and is present in trace levels in most capillary beds [9]. This effective location may ensure efficacious PC activation on the surface of large vessels where the ratio of the blood volume to the surface area is high.

Once activated, APC may either dissociate from EPCR, bind to its cofactor protein S [1,3], and exhibit its anticoagulant functions, or it may remain bound to EPCR and display cell-signaling cytoprotective activities [10–16] (Fig. 1a).

The clinical relevance of the PC pathway is evident from reports showing a clear association between deficiencies of PC [2,17,18] and protein S [19–21] or reduced APC levels [22–24] with thrombosis. In fact, the deficiencies of PC and protein S or the FV Leiden mutation are present in more than 50% of patients with inherited thrombophilia [25].

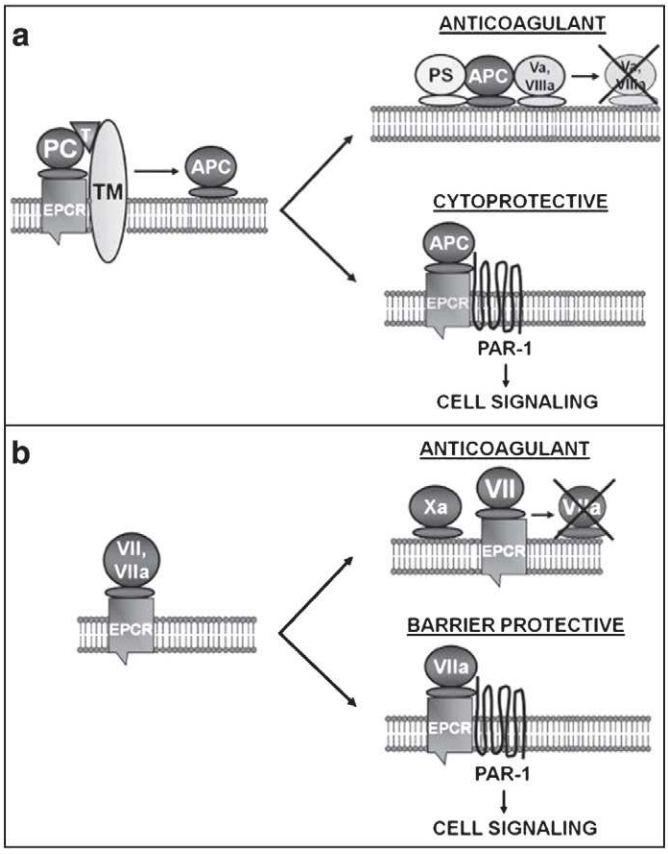

Fig. 1. Endothelial protein C receptor (EPCR) functions. **1a**. Protein C (PC) is activated by the thrombin (T)-thrombomodulin (TM)-EPCR ternary complex on the endothelial cell surface. Upon activation, activated PC (APC) may dissociate from the complex, bind to protein S (PS) and display its anticoagulant functions, or may remain bound to EPCR and display its cytoprotective activities through cell signaling mechanisms, most of them via protease-activated receptor-1 (PAR-1). **1b**. Factor (F) VII and FVIIa also bind to EPCR. Binding of FVII prevents the FXa-dependent generation of FVIIa, which may represent a new anticoagulant role for EPCR. On the other hand, FVIIa, upon binding to EPCR on endothelial cells, activates PAR1-mediated cell signaling and provides a barrier-protective effect.

EPCR is widely expressed on the surface of the endothelium of large vessels. Inflammatory stimuli-like tumor necrosis factor-α (TNF-α) or an atherosclerotic setting have been shown to reduce its expression [26,27]. The presence of EPCR on the surface of other cells has also been reported: trophoblasts, monocytes, neutrophils and eosinophils [28,29], brain endothelial cells [30], lymphocytes [31], osteoblasts [32], gastric epithelial cells [33], chondrocytes [34], tenocytes [35], epidermal keratinocytes [36], and human vascular smooth muscle cells [37]. More recently, EPCR was reported to be expressed in murine $CD8^+$ dendritic cells [38], hematopoietic stem cells [39], and cardiomyocytes [40].

EPCR structure

The human *EPCR* gene (*PROCR*) spans approximately 8 kb, is located on chromosome 20 at position q11.2, and consists of 4 exons [26,41–43]. Exon 1 [138 bp; 1 to 24 amino acids (aa)] encodes the 5′-untranslated region (UTR), the signal peptide, and 7 additional residues. Exons 2 (252 bp; 24 to 108 aa) and 3 (279 bp; 108 to 201 aa) encode most of the extracellular region of EPCR. Exon 4 (659 bp; 201 to 238 aa) encodes an additional 10 residues of the extracellular region of EPCR, the transmembrane domain, the cytoplasmic tail, and the 3′ UTR [42].

EPCR is a 46-kD type I transmembrane protein, constitutively expressed on the luminal surface of endothelium by endothelial cells, and is structurally similar to the major histocompatibility class 1/CD1 family proteins involved in the immune and inflammation responses [26]. The crystal structure of EPCR showed that a tightly bound phospholipid resides in the groove typically involved in antigen presentation, and its extraction results in loss of PC binding, which can be restored by lipid reconstitution [44]. The crystal structure, solved alone and in complex with the phospholipid-binding domain of protein C, revealed that most of the residues contacting the lipid in EPCR are identical to or highly conserved in CD1d, which may help to further understand the role of EPCR in immune regulation. Finally, it showed that the PC binding site is outside this conserved groove and is distal from the membrane-spanning domain [44].

The EPCR protein is comprised of 238 aa with a signal sequence at the amino-terminal end (17 aa); an extracellular domain composed of 2 alpha domains (α1 and α2), 4 potential N-glycosylation sites, and 3 Cys residues; a transmembrane domain near the carboxy-terminal (25 aa); and a short cytosolic domain (3 aa) [41,45,46].

Soluble EPCR and disease

A soluble form of EPCR (sEPCR), which lacks the transmembrane and cytoplasmic tail domain, is present in normal human plasma [47]. sEPCR is generated in vitro through proteolytic cleavage by metalloprotease activity inducible by thrombin and other inflammatory mediators, a process called shedding. This metalloprotease has been identified as the TNF-α converting enzyme or ADAM17 [48] and cleaves EPCR between aa's 192 and 200. Moreover, ADAM17 promotes the release of pro-inflammatory and adhesion molecules [48,49], and TNF-α significantly decreases the expression of EPCR and TM in several human endothelial cells [50]. A variety of mediators, including interleukin-1, hydrogen peroxide, phorbol esters, and thrombin dramatically increase EPCR shedding from the endothelium [51,52]. Like the membrane-bound form, sEPCR binds PC and APC with similar affinity. However, its binding to APC inhibits its anticoagulant and anti-inflammatory properties, and its binding to PC prevents PC activation by the thrombin-TM complex [53]. This would suggest that sEPCR may display procoagulant activity. sEPCR may also bind to activated neutrophils in a process that involves proteinase-3 and Mac-1. There is a controversy whether or not this interaction exerts an anti-inflammatory effect. On the one hand, it was proposed that binding of sEPCR to proteinase-3/Mac-1 may reduce leukocyte-

endothelial cells interactions, thus modulating inflammation and preventing endothelium damage [54,55]. However, it has also been reported that proteinase-3 is able to proteolyze EPCR, thus triggering an additional mechanism by which anticoagulant and cell protective pathways may be down-regulated during inflammation [56]. In sum, the physiological significance of sEPCR in vivo is not well known.

sEPCR levels showed a bimodal distribution in a healthy population. Approximately 15% to 20% of the general population has plasma sEPCR levels between 200 and 800 ng/mL, whereas the remainder have levels below 200 ng/mL [57]. Plasma sEPCR levels responded to anticoagulant treatment, suggesting that sEPCR may be a marker for a hypercoagulable state [58].

sEPCR levels increase in a wide variety of pathophysiological conditions, and may reflect endothelial dysfunction, and contribute to a procoagulant phenotype and increased risk of thrombosis. Accordingly, sEPCR levels increase in patients with sepsis, a disorder that results from a complex dysregulation of hemostatic mechanisms, with activation of procoagulant pathways and impairment of the fibrinolytic system and natural anticoagulant pathways, especially the PC pathway. In fact, as previously explained, the anti-inflammatory properties of APC seem to be beneficial in the treatment of sepsis [59,60]. sEPCR level also increases in systemic lupus erythematosus [61,62], a potentially fatal autoimmune disease affecting multiple organ systems, and it is associated with thrombotic manifestations, inflammation, and widespread activation of the vascular endothelium.

It has been shown that 50–80% of plasma sEPCR variations are under genetic control [63–65] and that most subjects with elevated sEPCR levels carry the H3 haplotype, one of the *EPCR* haplotypes described, but this will be discussed in detail later.

Recently, a new mechanism to generate sEPCR has been reported, consisting of the alternative mRNA splicing that generates a truncated EPCR mRNA lacking the sequence encoding the transmembrane and intra-cytoplasmic domains [66]. The resulting protein is not able to anchor to the membrane and, as a consequence, it is secreted and can be detected in plasma as sEPCR. The truncated sEPCR can bind PC and APC, and its generation is particularly efficient in H3-carrying subjects [66,67], and their endothelial cells might therefore be able to produce a soluble receptor that might compete with the membrane-bound EPCR for APC binding.

EPCR and FVII/FVIIa

It has been reported that EPCR may exhibit PC-independent anticoagulant activities. FVIIa is a serine protease that binds to tissue factor (TF) and initiates the coagulation cascade. The Gla domain of FVIIa exhibits an important degree of homology with the Gla domain of PC, and all the residues directly involved in the binding of PC to EPCR are conserved in FVII [68–70]. A binding analysis has shown that FVII, FVIIa, PC, and APC bind to EPCR with similar affinity[70–72], suggesting that the interaction of FVII/FVIIa with EPCR on endothelial cells may influence the activation of PC and APC-mediated cell signaling. In addition, EPCR mediates the internalization of FVIIa bound to it on the cell surface, indicating that it may play a role in FVIIa clearance [71,73,74].

More recently, it has been shown that endothelial cells may down-regulate the FXa-dependent generation of FVIIa through EPCR binding [75]. This regulation is probably made by moving FVII from phosphatidylserine-rich regions, suggesting a new anticoagulant role for EPCR. Moreover, FVIIa, upon binding to the EPCR on the endothelial cell surface, activates the endogenous protease-activated receptor-1 (PAR-1) and induces PAR-1-mediated p44/42 mitogen-activated protein kinase (MAPK) activation, thus providing a barrier-protective effect [76] (Fig. 1b).

It must be noted that, very recently, FXa has been shown to bind to EPCR, which opens new venues in serine-protease signaling [77].

Anti-EPCR autoantibodies

The presence of high titers of anti-EPCR autoantibodies has been described in patients with antiphospholipid syndrome, fetal death [78], deep vein thrombosis in the general population [79], and women with acute myocardial infarction [80]. Also, a case report described a patient with stroke and massive cutaneous necrosis who had high titers of anti-EPCR autoantibodies [81]. Two of these autoantibodies blocked the binding of PC to EPCR, and thus inhibited the generation of APC on the endothelium. [78]. For this reason, anti-EPCR autoantibodies may play a causative role in thrombosis, since low APC levels have been associated with an increased risk of venous and arterial thrombosis [24,82].

There is an association between elevated levels of the anti-EPCR autoantibodies, high levels of coagulation activity estimated by D-dimer levels, and levels of sEPCR [79], which could be related with endothelial injury induced by these autoantibodies. Anyhow, the mechanisms by which anti-EPCR autoantibodies confer a risk for thrombotic events are not fully understood.

EPCR polymorphisms and thrombosis

As mentioned before, normal APC generation depends on the precise assembly of thrombin and PC to their respective receptors, TM and EPCR, on the surface of endothelial cells. Any change in the efficiency of this coupling may cause altered APC generation and a modification in the risk of thrombosis. In fact, several mutations and polymorphisms have been reported in the *EPCR* gene, some of them associated with the risk of venous or arterial thrombosis.

EPCR and venous thromboembolism

Merati *et al.* [83] described a 23-bp insertion in exon 3. This mutation duplicates the preceding 23 bases and results in a STOP codon downstream from the insertion point [84]. Although statistical analysis did not reveal a significant association between the mutation and the risk of thrombosis, expression studies in mammalian cells showed that the truncated protein is not localized on the cell surface, cannot be secreted in the culture medium, and does not bind APC, suggesting that the insertion is a risk factor for arterial and venous thrombosis. However, given its low population frequency (<1%) [85,86], it is difficult to assess the effect of this mutation on the risk of venous and arterial thrombosis. In fact, after combining data from 7 studies that genotyped a total of 2,508 venous thromboembolism (VTE) patients and 2,617 controls [83–89], the prevalence of the mutation was 0.48% and 0.38%, respectively.

Hermida *et al.* described the point mutation C2769T, which causes a substitution of Arg to Cys at position 96 in the mature protein [90]. In vitro expression and characterization studies of the EPCR R96C variant revealed no biochemical differences with the wild-type counterpart, supporting no role of this mutation in VTE [90].

Up to 4 haplotypes of *EPCR* have been reported [63–65]: H1, H2, H3, and H4; 3 of which contain 1 or more single-nucleotide polymorphisms (SNPs) that are haplotype-specific (Fig. 2), while H2 contains the common allele of each SNP.

The H1 haplotype, tagged by the rare allele of 4678G/C (rs9574), has been associated with increased circulating APC levels [64] and a reduced risk of VTE in 2 independent studies [64,91]. H1 also reduced the risk of thrombosis in carriers of FV Leiden [92]. In patients with the FV Leiden mutation, the mean age at the first thrombosis was significantly higher in H1H1 propositi than in non-carriers of the H1 haplotype. In contrast, 2 other groups found no association of the H1 haplotype with the risk of thrombosis [63,65]. A possible explanation for the protective effect of *EPCR* H1 against VTE would be its association with increased APC levels. In fact, it has been described

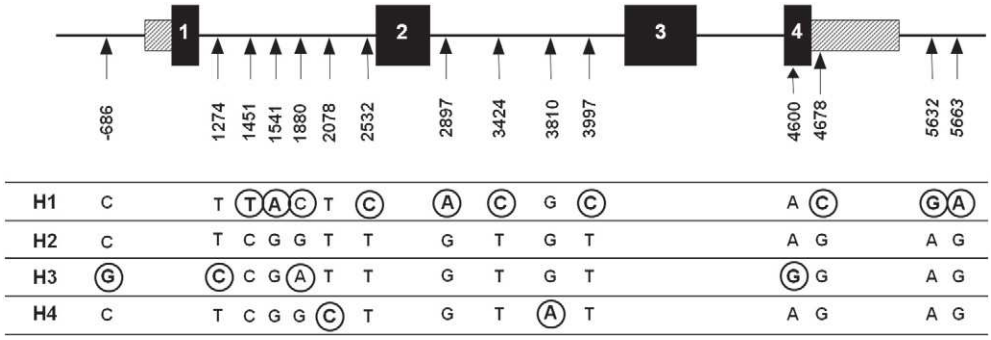

Fig. 2. The 4 haplotypes of the *EPCR* gene. Numbering according to Simmonds RE and Lane DA [42]. Circled letters correspond to specific alleles for each haplotype. Bold numbers indicate supposed numbering for these single-nucleotide polymorphisms according to this numbering since the sequence described by Simmonds RE and Lane DA do not reach these positions.

that a low level of APC in plasma is a strong, prevalent, independent risk factor for VTE [93,94].

EPCR is essential for normal embryonic development and plays a key role in preventing thrombosis at the maternal-embryonic interface [95]. Recently, it has been shown that the *EPCR* H1 seems to protect against recurrent pregnancy loss, particularly in carriers of FV Leiden [96], but not in the absence of this thrombophilic defect [97]. Presently it is unknown which SNP in *EPCR* H1 is responsible for the reported protective effect. The H1 haplotype contains 10 specific alleles, the 1451T (rs2069943), 1541A (rs2069944), 1880C (rs2069945), 2532C (rs2069948), 2897A (rs945960), 3424C (rs871480), 3997C (rs2069952), 4678C (rs9574), 5632G (rs1415773), and 5663A (rs1415774) (Fig. 2). Therefore, any of these nucleotides may be responsible for the observed association of H1 with increased levels of plasma APC and reduced risk of VTE [64,92]. Further studies are required to identify which polymorphism/s is responsible for the observed associations, although some efforts are being made [98].

The H3 haplotype, tagged by the rare allele of 4600A/G (rs867186), is associated with increased plasma levels of sEPCR, but its association with the risk of VTE is controversial [63–65,99]. One study [63] reported that carriers of the H3 haplotype have an increased risk of VTE in men but not in women, whereas others [64,65,91,99] did not find a significant association between *EPCR* H3 and the risk of thrombosis. The presence of *EPCR* H3 and concomitant elevated sEPCR plasma levels in carriers of the 2 dysfunctional PC variants, Arg-1Cys and Arg-1Leu, is associated with severe thrombotic manifestations [100]. In addition, it has been observed that *EPCR* H3 increases the risk of VTE in carriers of the prothrombin 20210A mutation, probably due to its association with increased sEPCR levels [101]. Furthermore, H3 carriers experienced the first VTE episode at a young age [101]. Recently, the maternal *EPCR* H3 allele has been found to be a mild risk factor for iliac VTE during pregnancy and puerperium [102]. Overall, the thrombogenicity of the *EPCR* H3, even if weak, does not seem anecdotal. First, the high incidence of this polymorphism in the Caucasian Mediterranean population (21.4%) and the fact that it may potentiate the prothrombotic effect of other thrombophilias, like the prothrombin 20210A allele [101], suggests that its contribution towards a VTE event may not be negligible. Second, the H3 haplotype may be a risk factor not only for VTE but also for pregnancy loss [103,104].

It has been suggested that high sEPCR levels associated with the H3 haplotype could be the mechanism that increases the thrombotic risk, even if sEPCR levels have not been deeply studied in this regard and some contradictory results exist [65,101]. Regarding the SNPs that comprise the H3 haplotype, the 4600G (219Gly) allele arises as the more obvious candidate responsible for the association of the H3 haplotype with increased sEPCR levels, in view of the fact that the cleavage of the EPCR anchored in the endothelial cell membrane to generate sEPCR occurs within the region of the protein encoded by exon 4, near the 4,600 position. The 4600G variant predicts a conformational change in the protein due to the Ser 219 to Gly substitution, which may render an EPCR more susceptible to cleavage by metalloproteases such as ADAM17 [48,63,64]. This hypothesis has been supported by 2 independent studies [105,106]. And other mechanism that could link the H3 haplotype and high levels of sEPCR is the alternative splicing previously explained [66]. The mean physiological sEPCR concentration is about 3 nM, well below the concentration of circulating PC, which is about 70 nM, and the Kd of the EPCR-PC/APC interactions is about 30 nM. It has been hypothesized that in individuals with markedly increased sEPCR levels due to the H3 haplotype (between 6 and 20 nM), the local concentration at the endothelial cell surface may approach or exceed the Kd value of the PC interaction, resulting in a decreased APC generation and inhibition of the APC generated [63].

Recently, a study that looked for genetic determinants for PC levels has shown that the *EPCR* H3 is associated with higher levels of plasma PC [107,108]. Additionally, the H3 haplotype has also been associated with higher levels of FVII [109,110], which could hypothetically confer its risk of thrombosis. An alternative explanation for the thrombogenicity that the H3 haplotype may induce is that the increased shedding of EPCR could reduce the amount of EPCR at the endothelial surface. In favor of this argument is the fact that inducing EPCR shedding in cells bearing the H3 haplotype notably reduced their ability to sustain PC activation as compared with non-H3 cells [106].

Finally, the *EPCR* H4 was reported to be associated with a slight increase in the risk of VTE [65], although no further studies have confirmed these results.

EPCR and arterial thrombosis

In accordance to the risk of VTE, the role of the *EPCR* 23-bp insertion [83] in the risk of arterial disease is hard to ascertain mainly due to the low prevalence of this mutation in the population. Actually, the combination of the data from the 3 reported studies that genotyped a total of 669 patients with myocardial infarction and 372 controls [83,84,111] revealed prevalences of 1.20% and 0.27%, respectively.

With regard to *EPCR* haplotypes and arterial disease, the results are also controversial. In the widest study performed so far, no association between the H3 haplotype and risk of coronary heart disease, stroke, or mortality was found [107]. In contrast, H1 was associated with lymphoid EPCR mRNA expression and with increased risk of incident stroke, all-cause mortality, and decreased healthy survival during follow-up [107]. These results are in discrepancy with the other wide study available, which showed that, surprisingly, both H1 and H3 haplotypes were associated with a reduction in the risk of premature myocardial infarction, and that these effects were additive [112]. The protective mechanism by which the H1 haplotype would reduce the risk of premature myocardial infarction may be related to an increase

in the circulating APC levels, which are characteristic of the carriers of the H1 haplotype [64,92]. The protection conferred by the H3 haplotype would be more difficult to explain as increased sEPCR levels would promote neither the activation of protein C nor the APC-dependent anticoagulant and cytoprotective actions. The rest of the studies available are not wide enough to withdraw powerful conclusions. One study [105] suggested that individuals carrying one *EPCR* H3 allele were protected from myocardial infarction, provided that they were neither diabetic nor showing symptoms of metabolic syndrome, since in patients with these pathologies, the H3 allele would increase the risk. Finally, there is another study which suggested that elevated sEPCR levels, linked to the H3 haplotype, might increase the risk of stroke at pediatric age [113].

Summary

EPCR plays an important role in the regulation of coagulation and in the APC-evoked cell signaling. Whether or not functional polymorphisms in the EPCR gene increase or decrease the risk of thrombosis, alone or in combination with other risk factors, has not been undoubtedly demonstrated and requires further investigation.

Conflict of interest statement

The authors state that they have no conflict of interest.

Acknowledgment

This work was supported by the PN de I+D+I 2008–20011 of Instituto de Salud Carlos III-Fondo de Investigación Sanitaria and FEDER (PS09/00610, PI10/01432 and Red Temática de Investigación RECAVA RD06/0014/0004 and RD06/0014/0008), Conselleria de Educación-Generalitat Valenciana (Prometeo/2011/027), and Fundación para la Investigación del Hospital Universitario La Fe (2007–0185), Spain. Pilar Medina is a Miguel Servet Researcher (ISCIII CP09/00065).

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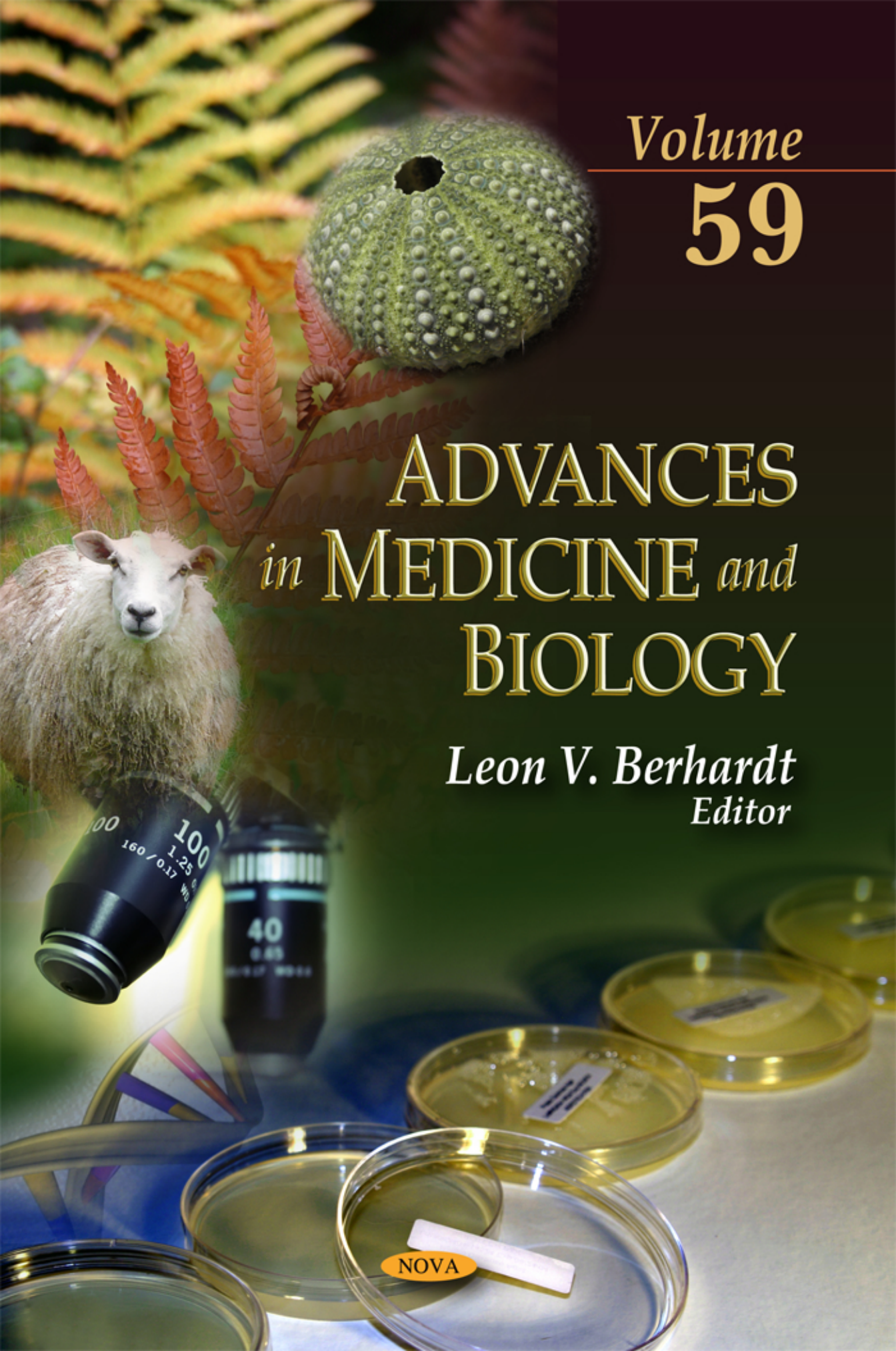
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VOLUME 59

LEON V. BERHARDT
EDITOR

NOVA BIOMEDICAL
New York

Library of Congress Cataloging-in-Publication Data

ISSN: 2157-5398

ISBN: 978-1-62257-836-8 (eBook)

Published by Nova Science Publishers, Inc. † New York

Contents

In: Advances in Medicine and Biology. Volume 59 ISBN: 978-1-62257-835-1
Editor: Leon V. Berhardt

Chapter 2

Anticoagulant and Cytoprotective Protein C Pathways: Clinical Implications

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Abstract

The protein C pathway plays a crucial role in the regulation of blood coagulation, as it controls the generation of thrombin. Protein C circulates in plasma as an inactive zymogen and is activated on the vascular endothelial cell membrane by the thrombin-thrombomodulin complex, a process further enhanced when protein C binds to its membrane receptor, the endothelial-cell protein C receptor (EPCR). Once generated, activated protein C (APC) can be released from this tetrameric activation complex and bind to protein S, which efficiently locates APC in the negatively charged phospholipidic surfaces where procoagulant reactions are taking place, and where APC inactivates factors (F) Va and VIIIa, ultimately inhibiting thrombin formation. Multiple proteins, including plasma protein S and FV, high density lipoprotein particles and lipids (such as phosphatidylserine, cardiolipin, phosphatidylethanolamine, and glycosphingolipids), act as APC cofactors to accelerate proteolysis of FVa and FVIIIa by APC. Effective APC generation depends on the precise assembly of thrombin, thrombomodulin, protein C and EPCR on the endothelial cell surface. Therefore, any change in the efficiency of this assembly may cause a reduction or increase in APC generation and modulate the risk of thrombosis.

On the other hand, once generated, APC can remain bound to EPCR on the endothelial cell surface and activate the protease activated receptor-1 (PAR1) to display cytoprotective functions. These APC-induced cell signalings, via EPCR and PAR1, link

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coagulation and inflammatory responses and can favorably regulate multiple pathways and cellular activities.

The physiological relevance of the protein C anticoagulant pathway is underscored by several observations: deficiencies of protein C or protein S are associated with an increased risk of venous thromboembolism (VTE), patients with the FV Leiden mutation show a resistance to APC and are also prone to VTE, decreased circulating APC levels have been associated with increased risk of VTE and myocardial infarction, and may contribute to the increased risk of VTE in some hypercoagulable states such as Behçet's disease or chronic renal failure. Additionally, APC exerts other cytoprotective properties, mainly mediated by antiinflammatory, antiapoptotic, neuroprotective, and barrier protection activities. Since EPCR and PAR1 are present on many cell types, including endothelial and epithelial cells, fibroblasts, myocites and neurons, APC could display cytoprotective effects in different cell types. Thus, although much remains to be understood regarding the biology of APC, preclinical studies suggest that APC has promising applications on several clinical settings such as: chronic wound healing, multi organ-failure caused by malaria, Dengue fever, ischemic stroke and other neurophatologies whose underlying pathologies involves deficits in the vasculo-neuronal-inflammatory triad, systemic lupus erythematosus nephritis, sepsis, premature atherosclerosis, inflammation in spinal cord injury, acute pancreatitis, acute lung injury, asthma, hepatic fibrogenesis, and severe ovarian hyperstimulation syndrome. Moreover, these properties of APC are currently leading to the development of APC molecules to be used as therapeutic tools.

This chapter summarizes the biological activities of the protein C pathway and its clinical implications.

1. Introduction

Blood coagulation takes place through a series of zymogen activations that culminate in thrombin generation and fibrin clot formation [1, 2]. Moreover, the haemostatic system comprises a group of pathways that intersect at several stages of the coagulation cascade inhibiting the coagulation process. Among these regulatory mechanisms of blood coagulation, the protein C (PC) pathway has emerged as crucial given its potential to participate in parallel antiinflammatory, antiapoptotic, and neuroprotective pathways.

The purpose of the PC pathway in haemostasis is to control thrombin formation in the area surrounding the clot. Once the haemostatic plug has filled the damaged area, which does not express thrombomodulin (TM), it begins to encroach upon the intact endothelium, where TM is expressed at high concentrations. Fibrin and TM compete for binding thrombin, and the high affinity of TM for exosite I of thrombin ensures the removal of thrombin from the clot, thus initiating the anticoagulant PC pathway [1]. By binding TM, the exosite I of thrombin is prevented from binding procoagulant substrates and this leads to allosteric changes in the reactive site that facilitate the cleavage of PC into activated PC (APC), especially when bound to the endothelial PC receptor (EPCR) in the quaternary complex thrombin-TM-PC-EPCR. The binding between PC and EPCR is Ca^{2+} and Mg^{2+}-dependent and involves the Gla domain of PC and a small clustered patch of residues on both α-helices of EPCR [3]. In the presence of protein S, APC inactivates factor (F) Va and FVIIIa, thus limiting thrombin generation on the clot periphery, where the endothelium remains intact. The proteolytic activity of APC is predominantly regulated by the PC inhibitor and to a lesser extent by the plasminogen activator inhibitor type 1 and α_1PI [4, 5].

In recent years a variety of new functions for the PC pathway have been described, all of them essentially cytoprotective. To carry out these functions, APC must activate PAR1 through a proteolytic cleavage mediated, generally, by EPCR. APC shows antiinflammatory properties by inhibiting the release of inflammatory mediators and decreasing the expression of vascular adhesion molecules, which results in an inhibition of leukocyte adhesion and infiltration. This is accomplished through the inhibition of the expression of the signaling molecules NF-κβ and p38 (which mediate the expression of TNFα and adhesion molecules), down-regulation of the expression of proinflammatory matrix metalloproteinase 3 (MMP-3), and up-regulation of antiinflammatory MMP-2. The antiapoptotic and neuroprotective functions of APC are carried out by diminishing p51, p53, Bax, caspase-3 and caspase-8 activity and up-regulating Bcl-2. Finally, APC has been shown to stabilize the endothelial cell barrier by converting sphingosine to sphingosine-1-phosphate [6, 7]. This wide spectrum of cytoprotective functions explains the recently reported role of the PC pathway in a variety of diseases such as severe sepsis, chronic wound healing, multi-organ failure caused by malaria, ischemic stroke, inflammation in spinal cord injury, acute pancreatitis, acute lung injury and asthma [6, 7], among others.

In the following sections both anticoagulant and cytoprotective functions of the PC pathway will be detailed.

2. Anticoagulant Role of the PC Pathway

The PC anticoagulant pathway plays a crucial role in the regulation of fibrin formation via proteolytic inactivation of the procoagulant cofactors FVa and FVIIIa [8-10]. PC is a vitamin K-dependent glycoprotein that is present in plasma at an average concentration of 70 nM. PC circulates in plasma as an inactive zymogen, which is activated to APC [11] on the surface of endothelial cells by the thrombin-TM complex. Another endothelial cell-specific protein, which is involved in the PC anticoagulant pathway, is the EPCR. EPCR binds PC on the endothelial cell surface. This binding enhances the rate of PC activation by the thrombin-TM complex by 10- to 20-fold [12] by decreasing the K_m of PC for its activation by this complex. EPCR is mainly expressed on the endothelium of large vessels, which counter-balances the relative low presence of TM and ensures an efficacious PC activation in these vessels. This occurs because EPCR is located in the lipid rafts of the membrane where it co-localizes with TM, and PC binding to EPCR through its Gla domain makes it much more accessible to the thrombin-TM complex.

Once activated, APC may either dissociate from EPCR, bind to its cofactor protein S [8, 10], and exhibit its anticoagulant functions, or it may remain bound to EPCR and display cell-signaling cytoprotective activities [5, 13-18] (Figure 1a).

The relevance of the anticoagulant role of EPCR *in vivo* has been well documented: EPCR blockade in baboons notably reduced the amount of APC generated upon thrombin administration [19], and an antiEPCR monoclonal antibody accelerated thrombus development in a murine model of thrombosis [20]. Additionally, EPCR may exhibit PC-independent anticoagulant activities. FVIIa is a serine protease that binds to tissue factor (TF) and initiates the coagulation cascade. The Gla domain of FVIIa exhibits an important degree of homology with the Gla domain of PC, and all the residues directly involved in the binding

of PC to EPCR are conserved in FVII [21-23]. Binding analyses showed that FVII, FVIIa, PC, and APC bind to EPCR with similar affinity [3, 23, 24], suggesting that the interaction of FVII/FVIIa with EPCR on endothelial cells may influence the activation of PC and APC-mediated cell signaling. In addition, EPCR mediates the internalization of FVIIa bound to it on the cell surface, indicating that it may play a role in FVIIa clearance [24-26].

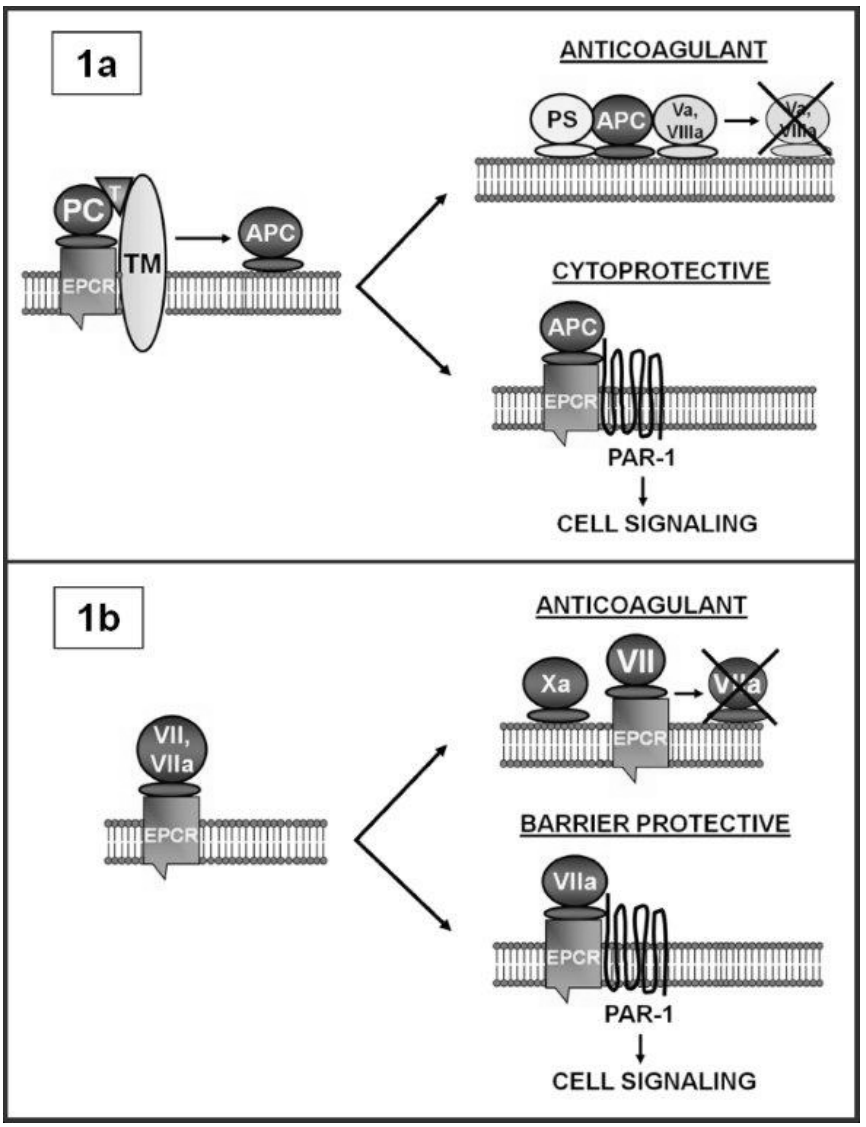

Figure 1. Functions of the protein C pathway. 1a. Protein C (PC) is activated by the thrombin (T)-thrombomodulin (TM)-EPCR ternary complex on the endothelial cell surface. Upon activation, activated PC (APC) may dissociate from the complex, bind to protein S and display its anticoagulant functions, or may remain bound to EPCR and display its cytoprotective activities through cell signaling mechanisms, most of them via protease-activated receptor-1 (PAR1). 1b. Factor (F) VII and FVIIa also bind to EPCR. Binding of FVII prevents the FXa-dependent generation of FVIIa, which may represent a new anticoagulant role for EPCR. On the other hand, FVIIa, upon binding to EPCR on endothelial cells, activates PAR1-mediated cell signaling and provides a barrier-protective effect. From Navarro S et al. [29].

More recently, it has been shown that endothelial cells may down-regulate the FXa-dependent generation of FVIIa through EPCR binding [27]. This regulation is probably made by moving FVII from phosphatidylserine-rich regions, suggesting a new anticoagulant role for EPCR. Moreover, FVIIa, upon binding to the EPCR on the endothelial cell surface, activates the endogenous protease-activated receptor-1 (PAR1) and induces PAR1-mediated p44/42 mitogen-activated protein kinase (MAPK) activation, thus providing a barrier-protective effect [28] (Figure 1b). It must be noted that, very recently, FXa has been shown to bind to EPCR, but whether it represents a high-affinity ligand of EPCR and, thus, shows any clinical relevance remains unsolved. Opposite results have been obtained for the signaling mechanisms triggered after FXa-EPCR binding on endothelial cells, suggesting that the relevance of the interaction between FX and EPCR is questionable [29].

A soluble form of EPCR (sEPCR), which lacks the transmembrane and cytoplasmic tail domain, is present in normal human plasma and generated by the shedding induced by metalloproteinase TNF-α converting enzyme/ADAM17 or by alternative splicing of its mRNA rendering a truncated EPCR lacking the sequence encoding the transmembrane and intra-cytoplasmic domains. A variety of mediators increase EPCR shedding from the endothelium. Moreover, ADAM17 promotes the release of proinflammatory and adhesion molecules, and TNF-α significantly decreases the expression of EPCR and TM in several human endothelial cells [29]. sEPCR binds PC and APC with the same affinity as the membrane-bound form. However, sEPCR binding to PC impairs PC activation and its binding to APC inhibits the APC anticoagulant and antiinflammatory properties. This would suggest that sEPCR may display procoagulant activity, although the physiological significance of sEPCR *in vivo* is not well known.

2.1. Lipids as Cofactors of the Anticoagulant APC

Although anionic charge on the phospholipid surface such as phophatidylserine (PS) is a necessary requirement for coagulation reactions to occur, it has become more evident that both major and minor neutral cell membrane lipids, such as cholesterol, cardiolipin or phosphatidylethanolamine (PE) and some glycosphingolipids, or plasma lipoproteins can positively influence anticoagulant processes, highlighting the potential role of membrane in enhancing APC anticoagulant activity.

Moyer et al. suggested that lipoproteins support prothrombinase and other procoagulant reactions [30]. Lipoproteins are the major carriers of plasma lipids and lipoprotein particles manifest multiple biologic activities. Several studies of plasma or serum lipids and lipoproteins have shown an association between dyslipidemia and VTE [31-34].

A well established atheroprotective role for HDL seems caused by multiple mechanisms such as reversion of cholesterol transport to the liver, decrease of the LDL oxidative damage, downregulation of the response of endothelial cells to adhesion molecules and inhibition of platelet aggregation. But HDL has other endothelial and antithrombotic actions. Deguchi et al. described that dyslipoproteinemia, especially low levels of large HDL particles and higher levels of small LDL particles, is associated with the occurrence of VTE in males under 55 years [35], and with the recurrence of VTE in adults after one episode of spontaneous VTE [36]. González-Ordóñez et al. reported an association between dyslipidemia and VTE, that was stronger in men than in women [37]. Among these studies, the association of

dyslipidemia with VTE was not as strong as observed for cardiovascular diseases, moreover this association was controversial.

In 1999 Griffin et al. reported for the first time that HDL promotes APC inactivation of FVa by enhancing the protein S-dependent APC cleavage of FVa or FV at Arg306. Moreover, they described a positive correlation between plasma apolipoprotein AI (apoA-I) levels and the anticoagulant response with the addition of purified HDL to an APC/protein S modified assay. They concluded that apoA-I, the major protein component of HDL particles, may be responsible for the anticoagulant cofactor activity of purified HDL, which provides evidence for a protective effect of high apoA-I levels for the risk of VTE. Particularly, *in vitro* studies showed that the best particle that exerts the anticoagulant activity is HDL_2 or large HDL [38]. In addition, an inverse correlation between HDL and plasma thrombin activation markers, such as prothrombin fragments F1+2 *in vivo* was found [39], suggesting that HDL may modify thrombin generation under normal conditions [40]. Recently, Oslakovic et al. described that HDL is unable to support the binding of FVa to its surface. They demonstrated that the enhancement of anticoagulant activities of APC and protein S observed associated with HDL is not a property of HDL, but rather is caused by contaminating negatively charged phospholipid membranes present in HDL prepared by ultracentrifugation. Thus, HDL enhances neither the prothrombinase reaction nor the APC-mediated degradation of FVa [41]. Deguchi et al. also speculate that the role of microdomains enriched in cholesterol and sphingolipids could be indirect, via helping to bind and localize APC where it may interact more efficiently with protein S to enhance FVa inactivation [42].

Pecheniuk et al. showed that cholesterol incorporation into phospholipid surfaces stimulates APC anticoagulant activity in plasma and enhances APC cleavage rates at R506 and R306 in FVa. Therefore, they suggested that cholesterol adds to the growing list of lipids that play a role in APC-dependent anticoagulant activity on the membrane surface [43].

In summary, patients with high levels of apolipoprotein AI, cholesterol, and large HDL particles have a decreased risk of recurrent VTE, via promotion of the anticoagulant action of the PC pathway. This finding may justify further clinical studies to assess whether strategies to elevate HDL using lifestyle changes or medication will reduce the risk of VTE.

Another antithrombotic action for HDL, indirectly related with the PC activation mechanism, was on the endothelium to minimize prothrombotic reactions via enhancement of endothelial nitric oxide synthase activity and via reduction of leukocyte adhesion to endothelium [38]. Coagulation and inflammation are intimately linked in the body's host-defense system, and inflammation may contribute to procoagulant processes and thus to both arterial and VTE. So, HDL has both direct and indirect antiinflammatory activities [44] that may effectively be translated into antithrombotic activities.

Moreover, studies of HDL infusions into cholesterol-fed rabbits indicated that HDL up-regulates endothelial cell thrombomodulin, which is indeed an additional anticoagulant factor that supports the generation of APC and suppression of thrombin generation [45]. This activity may have a variety of important implications, not only as enhancer of the PC pathway but as the antiinflammatory implications of TM [40, 46-48].

The metabolism of HDL is remarkably complex [49, 50]. The main participants in the reverse cholesterol transport pathway are principal contributors to HDL plasma levels. Mutations in the genes encoding ABCA1, LCAT, CETP, PLTP, HL, LPL, EL and SRB1 have an impact on HDL levels [51, 52]. Deguchi et al. analyzed single nucleotide polymorphisms (SNPs) in 3 key genes regulating HDL metabolisms, hepatic lipase,

endothelial lipase and cholesterol ester transfer protein (CETP) [43]. They found that the dyslipoproteinemia in male with VTE is associated with differences in CETP genotypes. A common TaqI B1 variation in the CETP gene was associated with elevated plasma CETP and an unfavourable pattern of lipoproteins, fact that may contribute to the dyslipoproteinemia observed in these VTE patients [53].

CETP plays a pivotal role in lipoprotein metabolism and remodels HDL by transferring lipids out of HDL into other lipoprotein particles or membranes. CETP deficiency or CETP inhibitors increase HDL levels, mainly due to increasing levels of large HDL particle levels. The CETP gene and its association with lipoprotein concentrations as well as with CETP mass and activity have been related to dozens of SNPs and several haplotypes [54, 55].

The association of phospholipids with coagulation reactions has been extensively studied *in vitro*. These studies showed that PE and PS exhibit significant influences on the blood coagulation reactions and might contribute to either beneficial or deleterious properties of lipoproteins. PS is generally described as the most procoagulant phopholipids in membranes [56, 57], whereas PE was reported to support activated PC anticoagulant activity [58, 59]. Another phospholipid, cardiolipin, present as a normal component of human plasma lipoproteins [60], has been shown to stimulate the anticoagulant PC pathway by increasing the affinity of phospholipid surfaces for protein S:APC and by enhancing inactivation of FVa by APC due to cleavages at Arg506 and Arg306 [61]. Based on this, it was further hypothesized that anti-cardiolipin or anti-oxidized cardiolipin antibodies may be thrombogenic because they may inhibit phospholipid-dependent expression of the anticoagulant PC pathway [60, 61].

The relationships between sphingolipids, especially neutral sphingolipids, and coagulation reactions or thrombotic events have not been very well explored. However, some findings have been reported. Glucosylceramide (GlcCer) and related neutral glycosphingolipids are anticoagulant lipid cofactors for activated PC anticoagulant actions [42, 62-64], and low plasma levels of GlcCer are associated with VTE [42, 65]. The addition of GlcCer to PC/PS vesicles enhanced protein S-dependent APC cleavage in FVa at Arg 506 by 13-fold, whereas PC/PS vesicles alone minimally affected protein S enhancement of this reaction [63]. Moreover, addition of GlcCer into PC/PS vesicles prolonged FXa clotting assays in the presence of APC, suggesting that GlcCer enhances anticoagulant but not procoagulant reactions in plasma [62].

Furthermore, the use of either second- or third-generation oral contraceptives decreases plasma GlcCer levels and this reduction correlates with a reduction of plasma sensitivity to the anticoagulant proteins, APC/protein S [66]. These findings support the hypothesis that the effect of oral contraceptives on GlcCer metabolism could cause a reduction in the sensitivity of plasma to the PC anticoagulant pathway, thereby increasing VTE risk.

On the other hand, sphingosine at concentrations similar to those found in plasma inhibits prothrombin activation on platelet surfaces and in model coagulation assay systems. Mechanistically, the anticoagulant action of sphingosine is apparently mediated through the inhibition of the procoagulant interactions between purified FXa and FVa. Thus, sphingosine may directly downregulate thrombin generation [62].

The anticoagulant sphingolipids GlcCer and sphingosine are dynamically metabolically related to ceramide. Ceramide is one of the most studied sphingolipids and it contributes to the induction of apoptosis that may be implicated in endothelial cell damage and, thereby, the expression of procoagulant activities. The dynamic metabolic balances among various

sphingolipid metabolites may be shifted by a variety of factors. Changes in the relative concentrations of sphingolipids might regulate inflammatory events such as cell proliferation and also might down-regulate blood coagulation and thrombin generation. Various enzymes that regulate sphingolipid metabolism are capable of shifting the balance between levels of sphingosine and ceramide, sphingosine and sphingosine-1-phosphate, GlcCer and ceramide, etc. Moreover, it has been suggested that certain sphingolipid metabolic imbalances could be causally associated with VTE [40].

2.2. Clinical Implications of the PC Pathway in Thrombotic Pathologies

The anticoagulant PC pathway is vital to the maintenance of normal haemostasis. The clinical relevance of the PC pathway is evident from reports showing a clear association between deficiencies of PC [9, 67, 68] and protein S [69-71] with thrombosis. Moreover, reduced circulating APC levels are an independent risk factor for both venous and arterial thrombosis [72-75]. In fact, deficiencies of PC and protein S or the FV Leiden mutation are present in more than 50% of patients with inherited thrombophilia [76].

The presence of high titers of anti-EPCR autoantibodies has been described in patients with antiphospholipid syndrome and are a risk of fetal death [77], deep vein thrombosis [78], and young women with acute myocardial infarction (MI) [79]. Also, a case report described a patient with stroke and massive cutaneous necrosis who had high titers of anti-EPCR autoantibodies [80]. Two of these autoantibodies blocked the binding of PC to EPCR, and thus inhibited the generation of APC on the endothelium. [77]. These reports suggest that anti-EPCR autoantibodies may play a causative role in thrombosis, as low APC levels have been associated with an increased risk of venous and arterial thrombosis [72, 75]. There is an association between elevated levels of the anti-EPCR autoantibodies, high levels of coagulation activity estimated by D-dimer levels, and levels of sEPCR [78], which could be related with endothelial injury induced by these autoantibodies. Anyhow, the mechanisms by which anti-EPCR autoantibodies confer a risk for thrombotic events are not fully understood.

sEPCR levels increase in a wide variety of pathophysiological conditions, and may reflect endothelial dysfunction, and contribute to a procoagulant phenotype and increased risk of thrombosis. Accordingly, sEPCR levels increase in patients with sepsis, a disorder that results from a complex dysregulation of haemostatic mechanisms, with activation of procoagulant pathways and impairment of the fibrinolytic system and natural anticoagulant pathways, especially the PC pathway. In fact, as further discussed, the antiinflammatory properties of APC seem to be beneficial in the treatment of sepsis [81, 82]. sEPCR level also increases in systemic lupus erythematosus [83, 84], a potentially fatal autoimmune disease affecting multiple organ systems, and it is associated with thrombotic manifestations, inflammation, and widespread activation of the vascular endothelium.

As mentioned before, normal APC generation depends on the precise assembly of thrombin and PC to their respective receptors, TM and EPCR, on the surface of endothelial cells. Any change in the efficiency of this coupling may cause altered APC generation and a modification in the risk of thrombosis. As previously mentioned, EPCR is a key protein of the PC pathway. In fact, several polymorphisms have been reported in the *PROCR* gene, some of them associated with the risk of venous or arterial thrombosis.

Up to 4 haplotypes of *PROCR* have been reported [85-87]: H1, H2, H3, and H4; 3 of which contain 1 or more SNPs that are haplotype-specific (Figure 2), while H2 contains the common allele of each SNP.

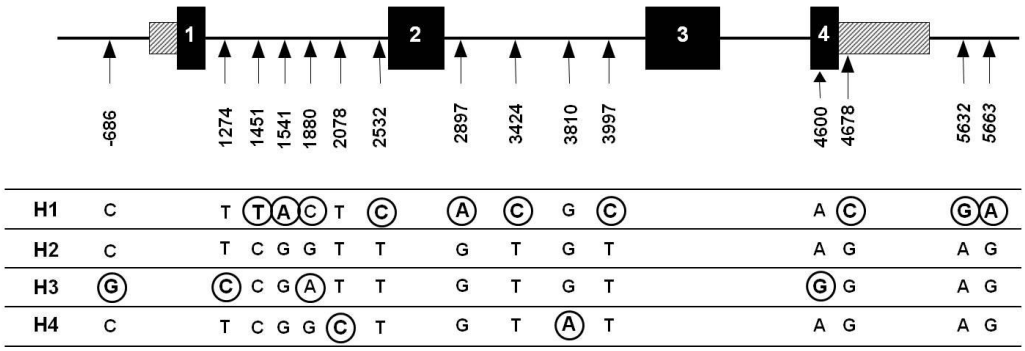

Figure 2. The 4 haplotypes of the EPCR gene. Numbering according to Simmonds RE and Lane DA [166]. Circled letters correspond to specific alleles for each haplotype. Bold numbers indicate supposed numbering for these single-nucleotide polymorphisms according to this numbering since the sequence described by Simmonds RE and Lane DA do not reach these positions. From Navarro S et al. [29].

The H1 haplotype, tagged by the rare allele of g.4678G>C (rs9574), has been associated with increased circulating APC levels [86] and a reduced risk of VTE in 2 independent studies [86, 88]. H1 also reduced the risk of thrombosis in carriers of FV Leiden [89]. In patients with the FV Leiden mutation, the mean age at the first thrombosis was significantly higher in H1H1 propositi than in non-carriers of the H1 haplotype. Recently, a study evaluating the association of the *PROCR* haplotypes with the risk of VTE in Behçet´s disease, a rare autoimmune disease associated with vasculitis and a moderate prevalence of concomitant thrombosis, showed that the H1 protects Behçet´s patients from VTE [90]. Furthermore, increased APC levels apparently protect these patients from developing posterior uveitis. In contrast, 2 other groups found no association of the H1 haplotype with the risk of thrombosis [85, 87]. A possible explanation for the protective effect of *PROCR* H1 against VTE would be its association with increased APC levels. In fact, it has been described that a low level of APC in plasma is a strong, prevalent, independent risk factor for VTE [72, 91].

EPCR is essential for normal embryonic development and plays a key role in preventing thrombosis at the maternal-embryonic interface [92]. Moreover, it has been shown that the *PROCR* H1 seems to protect against recurrent pregnancy loss, particularly in carriers of FV Leiden [93], but not in the absence of this thrombophilic defect [94]. A recent study evaluated the effect of EPCR on PC activation *in vivo*. It is known that oral anticoagulants (OAC) reduce plasma APC levels in a lesser extent than those of PC in lupus erythematosus and cardiac patients, and this effect was also confirmed for VTE patients that was much more pronounced in homozygous carriers of the *PROCR* H1 haplotype [95]. The results of this study suggest that the H1 haplotype is a major determinant for this effect (possibly via the higher APC levels associated to this H1), which may have clinical implications. Furthermore, part of the PC molecules in OAC patients will be sub- or non-carboxylated, but this fraction of PC without Gla residues cannot be activated *in vivo*. The PC Gla domain is essential for

binding to EPCR, and a sufficiently carboxylated PC Gla-domain is essential for *in vivo* PC activation, which indirectly suggest that EPCR is necessary for this activation [95].

Presently, it is unknown which SNP in *PROCR* H1 is responsible for the reported protective effect. The H1 haplotype contains 10 specific alleles, the g.1451T (rs2069943), g.1541A (rs2069944), g.1880C (rs2069945), g.2532C (rs2069948), g.2897A (rs945960), g.3424C (rs871480), g.3997C (rs2069952), g.4678C (rs9574), g.5632G (rs1415773), and g.5663A (rs1415774) (Figure 2). Therefore, any of these nucleotides may be responsible for the observed association of H1 with increased levels of plasma APC and reduced risk of VTE [86, 89]. Further studies are required to identify which polymorphism/s is responsible for the observed associations, although some efforts are being made [96].

The H3 haplotype, tagged by the rare allele of g.4600A>G (rs867186), is associated with increased plasma levels of sEPCR, but its association with the risk of VTE is controversial [85-87, 97]. One study [85] reported that carriers of the H3 haplotype have an increased risk of VTE in men but not in women, whereas others [86-88, 97] did not find a significant association between *PROCR* H3 and the risk of thrombosis. A recent study revealed that carriers of *PROCR* H3 are protected against the clinical manifestations associated with Behçet's disease, and that this protection is likely due to the moderately increased levels of sEPCR that are associated with this haplotype [90]. The presence of *PROCR* H3 and concomitant elevated sEPCR plasma levels in carriers of the 2 dysfunctional PC variants, p.Arg-1Cys and p.Arg-1Leu, is associated with severe thrombotic manifestations [98]. In addition, it has been observed that *PROCR* H3 increases the risk of VTE in carriers of the prothrombin g.20210A mutation, probably due to its association with increased sEPCR levels [99]. Furthermore, H3 carriers experienced the first VTE episode at a young age [99]. Additionally, the maternal *PROCR* H3 allele has been found to be a mild risk factor for iliac VTE during pregnancy and puerperium [100]. A recent meta-analysis in which 12 candidate genes and 13 genome-wide association studies were analyzed, showed that the risk of VTE significantly increases by a factor of 1.22 (95% confidence interval, 1.11-1.33, $P<0.001$) for every additional copy of the G allele [101]. Overall, the thrombogenicity of the *PROCR* H3, even if weak, does not seem anecdotal. First, the high incidence of this polymorphism in the Caucasian Mediterranean population (21.4%) and the fact that it may potentiate the prothrombotic effect of other thrombophilias, like the prothrombin g.20210A allele [99], suggests that its contribution towards a VTE event may not be negligible. Second, the H3 haplotype may be a risk factor not only for VTE but also for pregnancy loss [102, 103].

The high sEPCR levels associated with the H3 haplotype might be responsible for the increase in the thrombotic risk. Among the SNPs comprised in the *PROCR* H3, the g.4600G (p.219Gly) allele arises as the more obvious candidate responsible for the association of the H3 haplotype with increased sEPCR levels, in view of the fact that the cleavage of the membrane-bound EPCR to generate sEPCR occurs around its position in exon 4. The Ser 219 to Gly substitution predicts a conformational change in the protein rendering an EPCR more susceptible to cleavage by ADAM17, leading to a truncated mRNA through alternative splicing [104].

Recently, a study that looked for genetic determinants for PC levels has shown that the *PROCR* H3 is associated with higher levels of plasma PC [105, 106]. Additionally, the H3 haplotype has also been associated with higher levels of FVII [107, 108], which could hypothetically confer its risk of thrombosis. An alternative explanation for the thrombogenicity that the H3 haplotype may induce is that the increased shedding of EPCR

could reduce the amount of EPCR at the endothelial surface. In favor of this argument is the fact that inducing EPCR shedding in cells bearing the H3 haplotype notably reduced their ability to sustain PC activation as compared with non-H3 cells [109].

Finally, the *PROCR* H4 was reported to be associated with a slight increase in the risk of VTE [87], although no further studies have confirmed these results.

With regard to *PROCR* haplotypes and arterial disease, the results are also controversial. In the widest study performed so far, no association between the H3 haplotype and risk of coronary heart disease, stroke, or mortality was found [105]. Supporting these findings, the aforementioned recent meta-analysis also failed to demonstrate an association between H3 and increased risk of MI [101]. In contrast, H1 was associated with lymphoid *PROCR* mRNA expression and with increased risk of incident stroke, all-cause mortality, and decreased healthy survival during follow-up [105]. These results are in discrepancy with the other wide study available, which showed that both H1 and H3 haplotypes were associated with a reduction in the risk of premature MI [110]. The protective mechanism by which the H1 haplotype would reduce the risk of premature MI may be related to an increase in the circulating APC levels, which are characteristic of the carriers of the H1 haplotype [86, 89].

3. Cytoprotective Role of the PC Pathway

Increasing evidence links blood coagulation proteins with the regulation of acute and chronic inflammatory disease. Particularly interesting are vitamin K-dependent proteases, such as PC, which are generated as a haemostatic response to vascular injury, but can also modulate the immune response upon infection or injury [111]. These immune-regulatory effects require initiation of intracellular signaling networks via interaction with vascular receptors. In some cases, this interaction alone is sufficient to trigger signal transduction but, more commonly, extracellular receptor interactions position PC so that protease-sensitive receptor activation and intracellular signal transduction can occur. These receptors are a family of cleavage-sensitive G-protein coupled receptors, the so-called PARs [112]. Furthermore, PAR1 activation commonly occurs upon presentation of the PC protease by a proximal coagulation protein co-receptor, and EPCR fulfils this role for several vitamin K-dependent coagulation factors. Moreover, EPCR blockade prevents APC activation of PAR1 [13]. Not surprisingly, EPCR has arisen as a key protein which modulates the cytoprotective properties of APC in a constant increasing number of inflammatory disorders.

Traditionally, PAR1 was known as the receptor engaged by thrombin to trigger cell signaling mechanisms leading to proinflammatory, proadhesive, barrier disrupting and proapoptotic actions. The affinity of thrombin for PAR1 is several orders of magnitude higher than that exhibited by APC, and thrombin is needed for APC to be generated. Therefore, is not totally understood how the endogenously generated APC is able to induce PAR1-dependent mechanisms, and why such mechanisms are opposite to those exerted by thrombin when both of them activate the same mediator [113]. One plausible explanation is that EPCR, when not occupied by a ligand, may remain associated with caveolin-1, which is also present in the rafts. Under these conditions the thrombin-dependent activation of PAR1 would elicit a proinflammatory signal through $G_{12/13}$ and G_q coupling. However, the occupancy of EPCR by APC or even PC, would disrupt the association between EPCR and caveolin-1. Subsequently,

PAR1 would couple G_i instead of $G_{12/13}$ and G_q, and an antiinflammatory expression pattern would be triggered [114]. Additional data derived of experiments performed on the surface of endothelial cells using APC and thrombin simultaneously, showed that while thrombin-activated PAR1 was rapidly internalized and degraded, APC-activated PAR1 remained on the cell surface. This would mean that although the rate of thrombin-PAR1 cleavage might be much higher, the APC-cleaved receptor would accumulate on the surface mediating relevant signaling even in the presence of thrombin [115]. Conversely, the complete mechanism responsible for the APC-PAR1 protective signaling still remains unexplained. Recently, Schuepbach et al. have suggested an alternative explanation. They provided a novel concept on how APC and thrombin mediate distinct effects, proposing that the enzyme-specific cleavage sites induce specific conformations which mediate divergent downstream effects. This unexpected model of PAR1 signaling might lead to novel therapeutic options for treatment of inflammatory diseases [116].

APC binding to EPCR enables PAR1 activation and APC-specific PAR1/G-protein coupling [13], but not PAR2 activation on endothelial cells. However, APC has been shown to activate PAR2 on EPCR/PAR2 transfected cells, and PAR1 cleavage by APC may initiate PAR2 activation by a trans-activation mechanism currently unknown [117, 118]. The cellular microenvironment has revealed crucial for EPCR-dependent PAR1 signaling by APC. Chemical disruption of lipid rafts disables EPCR-dependent protective PAR1 signaling by APC against thrombin-induced endothelial cell barrier permeability and TNF- α-induced apoptosis in endothelial cells [15]. PAR1 recruitment with EPCR to caveolin-rich microdomains alters G-protein coupling specificity, and thus the downstream signaling response upon PAR1 activation [114]. Moreover, caveolae formation is crucial for EPCR-dependent APC signaling via PAR1 [119].

Furthermore, the involvement of other cell-receptors such as Tie2, S1P1, CD11b/CD18, ApoER2 (LRP8) and epidermal growth factor receptor in APC signaling has been recently shown, thereby providing yet additional insights into the molecular mechanism that underlies the cell-protective properties of APC [120-122].

Finally, EPCR-bound APC is able to initiate antiinflammatory signal transduction pathways in a PAR1-independent manner on myeloid cells. Apolipoprotein E receptor 2-bound APC induces adaptor protein disabled-1 and glycogen synthase kinase 3β phosphorylation via PI3 K/Akt signaling pathway on monocytic U937 cells [120], thereby contributing to APC's beneficial effects on cells.

Overall, these results lead to the fact that APC induces multiple cytoprotective benefits, including down-regulation of injury-induced vascular barrier permeability both *in vitro* [123] and *in vivo* [124], down-regulation of inflammatory cytokines (IL-6, TNF-α) [125, 126] and up-regulation of IL-10 [127]. Furthermore, APC-EPCR-dependent PAR1 activation attenuates apoptosis in both endothelial cells [128] and neurons [129].

3.1. Clinical Implications of the PC Pathway in Inflammatory Pathologies

The widely observed beneficial effects of APC in animal models of sepsis, pulmonary injury, stroke and wound healing cannot be explained only by APC’s anticoagulant effects. Furthermore, important recent studies have suggested that APC administration may have therapeutic applications in a rage of diverse inflammatory settings that will be discussed in

more detail. As previously described, EPCR binding is required for the beneficial effects of APC in each of these disease models. In fact, increasing evidence suggests that diminished EPCR expression and/or function can be a contributory factor in conditions characterized by chronic inflammation and autoimmune disease. For instance, individuals with active inflammatory bowel disease exhibit depleted EPCR expression and increased EPCR shedding on their colonic mucosal microvasculature, caused by local generation of TNF-α and IL-β in the inflamed local environment [130].

Patients with sepsis show intravascular and extravascular fibrin formation, suggesting that the activation of coagulation and the inhibiton of fibrinolysis are important mechanisms in the pathogenesis of sepsis. PC is consumed during the development of severe septic shock and this decrease may further contribute to the development of microvascular thrombosis and disseminated intravascular coagulation. Moreover, the cytokine response in sepsis results in decreased expression of TM and EPCR on the endothelium and, thus, in a decrease in PC activation. Several studies in animal models showed that blockade or inhibition of PC activation [131], EPCR binding to PC or APC [19, 132], or protein S [131, 133] exacerbates the septic response. In contrast, administration of PC in a sepsis model of baboon results in prevention of shock and disseminated intravascular coagulation [134]. These results suggested that the downregulation of the anticoagulant PC pathway plays an important role in the modulation of coagulation and inflammation in sepsis [135], and that APC administration might be beneficial for the treatment of sepsis in humans. Moreover, the signaling of the exogenously administered or endogenously generated APC has been shown to be critical in protection from lipopolysaccharide-induced septic shock or acute lung injury in animal models [136]. In order to evaluate the potential benefit of APC in the treatment of sepsis, the PROWESS and RESPOND clinical trials were developed [137]. APC initially succeeded in reducing severe sepsis-associated mortality [138] in fact, it has been the only drug approved by the American Food and Drug Administration for the treatment of severe sepsis. However, very recently the company that owns the rights to APC for clinical use has withdrawn it from the market since a recent study has questioned the efficacy of recombinant human activated protein C, or drotrecogin alfa (activated), for the treatment of patients with septic shock [139], probably due to dose adjustment.

Antiinflammatory, antithrombotic, and neuroprotective effects mediated via the APC-EPCR-PAR1 axis were also found in a model of ischaemic stroke [140]. In addition, APC inhibited apoptosis in hypoxic human brain endothelium by transcriptionally-dependent inhibition of the tumor suppressor p53, normalization of the Bax/Bcl-2 ratio and reduction of caspase 3 signaling [18]. Moreover, APC has been found to be neuroprotective, both in a stroke model in mice and in cultured cortical neurons, with the effects being both EPCR-dependent and PAR1–dependent [141]. Interestingly, in the same mouse model of stroke, the administration of protein S was likewise found to provide neuroprotection [142]. Furthermore, APC extended the time window of tissue-plasminogen activator (t-PA)-dependent thrombolytic therapy [143]. APC blocked the t-PA-induced apoptosis and the nuclear factor kappa B-dependent MMP-9 pathway in ischemic brain endothelium [144, 145]. The exacerbated activity of MMP-9 leads to the loss of integrity of the blood brain barrier and accounts for the non-desired prohaemorrhagic side effect of t-PA. Thus, the EPCR-dependent APC actions would contribute not only to reduce the lesion size but, interestingly, to reduce the t-PA-induced brain haemorrhage [145]. Particularly promising is the role that pharmacologically administered APC may play in the near future in stroke treatment, as

shown in several murine stroke models. A preclinical study using a modified APC molecule points out at APC as a feasible therapy for ischemic stroke patients [146]. Moreover, a clinical trial evaluating the efficacy of APC treatment in ischaemic stroke is currently being carried out (clinical trial identifier NCT00533546; http://www.clinicaltrials.gov).

Although APC has failed to be effective for the treatment of diseases such as septic shock, APC has been successfully used in other inflammatory diseases such as colitis, Dengue shock syndrome and systemic lupus erythematosus. Scaldaferri F et al. observed that the use of APC ameliorated experimental colitis in mice in an EPCR-dependent manner [130]. In addition, Vetrano S et al. [147] have demonstrated that the PC pathway is a unique system involved in controlling intestinal homeostasis and inflammation by regulating epithelial barrier function. Thus, PC(-/-)/PC(Tg) mice, expressing only 3% of WT PC, developed spontaneous intestinal inflammation and were prone to severe experimental colitis. These mice also demonstrated spontaneous elevated production of inflammatory cytokines and increased intestinal permeability. *In vitro*, treatment of epithelial cells with APC led to protection of tight junction disruption induced by TNF-α, and *in vivo*, topical treatment with APC led to mucosal healing and amelioration of colitis.

Dengue fever infection is the most prevalent viral disease in humans. No safe vaccine, no experimental animal model, and no specific antiviral treatment are available. Clinical observations revealed abnormalities in the coagulation and inflammatory systems. In fact, recent studies present novel insights into the participation of Dengue fever in the downregulation of the thrombin-TM-PC complex formation on the endothelial surface, which produces a reduction in APC generation and may explain the vasculophathy observed during the shock syndrome [148]. Therefore, further research on the mechanisms that underlie the alterations in the anticoagulant and cytoprotective PC pathway in this disease may help to the design of new therapeutic strategies for Dengue Fever.

Recent studies described an effect of EPCR to antagonize and limit the endothelial damage of systemic lupus erythematosus nephritis, acting as a repair component [149]. A usefulness of APC for the treatment of systemic lupus erythematosus has also been suggested after it was reported that APC succeeded in suppressing the abnormal systemic immune activation in systemic lupus erythematosus mice, probably through both EPCR-dependent and EPCR-independent mechanisms [150]. Also, the antiapoptotic effects of APC have also been shown to protect against diabetic nephropathy by acting on endothelial cells [151], and on podocytes via PAR-3 [152].

Recently, Xue M et al. [153] have reported that, when human recombinant APC was delivered intraperitoneally to non-obese diabetic mice, it inhibited pancreatic islet inflammation, stimulated T regulatory cells, and prevented diabetes. The APC mechanism of action seemed complex, involving induction of T regulatory cells differentiation, inhibition of inflammation, and possibly direct cytoprotective effects on β cells.

APC can also mediate multiple neuroprotective benefits, which are dependent upon its interaction with EPCR on central nervous system cells and the blood-brain barrier. In fact, The APC–EPCR complex formation is required for APC transcytosis across the blood–brain barrier and delivery into the cerebrospinal fluid to achieve the central nervous system [154]. Recent studies showed that APC can favorably regulate multiple pathways within the neurovascular unit in non-neuronal cells and neurons during acute chronic central nervous system events, leading to stabilization of the blood-brain barrier, neuroprotection and control of neuroinflammation. In detail APC enhances brain endothelial barrier integrity [155],

stimulates angiogenesis [156, 157], reduces the passage of neurotoxic circulatory proteins into the central nervous system [158] and leukocyte migration across the blood–brain barrier [140]. APC also directly inhibits microglial inflammation via activation of PAR1 [158] and neuronal cell apoptosis via PAR1 and PAR3 activation [141, 159, 160]. These preclinical studies suggest that APC has promising applications on neuropathologies whose underlying pathology involves deficits in the vasculo-neuronal-inflammatory triad [161], and highlight a potential role for non-anticoagulant APC as a drug target for these diseases [162].

The cytoprotective role of the APC-EPCR axis also seems to play a role in cancer. EPCR is expressed in several cancer cell lines including monoblastic leukemia, glioblastoma, osteosarcoma, erythroleukemia, and prostate cancer cells [163]. Interestingly, EPCR can dictate divergent cancer cell behaviors: EPCR-dependent APC cleavage of PAR1 increases invasion and chemotaxis of breast cancer cells, without alteration of cell proliferation [164]. In contrast, $EPCR_{HIGH}$ mice with melanoma metastasis are less prone to metastatic infiltration into both the lungs and liver compared to wild-type mice. *In vitro* analysis of APC-treated cultured B16-F10 melanoma cells indicate that this function is a consequence of the APC-EPCR complex-dependent down-regulation of tumor adhesion and transendothelial migratory functions [17].

All of the above referenced actions do not require the anticoagulant activity of APC. In fact, one of the major limitations of APC as a therapeutic drug is the concomitant hemorrhagic episodes. Therefore, in the last years many efforts are being made to develop APC molecules which retain its cytoprotective activities but lose its anticoagulant activity (nicely reviewed in [165]). This engineered APC molecules emerge as potent therapeutic tools for the near future, since they could presumably be administered at higher doses thus improving the clinical efficiency.

Conclusion

The PC anticoagulant pathway is vital to maintain normal haemostasis. The purpose of the PC pathway in haemostasis is to control thrombin formation in the area surrounding the clot. Among these regulatory mechanisms of blood coagulation, the PC pathway has emerged as crucial given its potential to participate in parallel antiinflammatory, antiapoptotic, and neuroprotective pathways.

Among the molecules involved in the PC pathway, EPCR has emerged as a central player in the convergent pathways of haemostasis and inflammation. EPCR enhances PC activation up to 20 times, which makes it essential for the PC anticoagulant properties. Furthermore, EPCR binding unlocks the signaling potential of APC and potentially other ligands on numerous cell types, initiating down-regulation of multiple inflammatory processes. EPCR interaction is central to many of the potential therapeutic applications proposed for recombinant APC, and its prominent role in regulating endogenous response to infection has been unveiled in numerous animal models of inflammatory disease.

In recent years a variety of new functions for the PC pathway have been described, all of them essentially cytoprotective. This wide spectrum of cytoprotective functions explains the recently reported role of the PC pathway in a variety of diseases such as severe sepsis, ischemic stroke, colitis, Dengue shock syndrome, systemic lupus erythematosus, pancreatic

islet inflammation, and other neurophatologies whose underlying pathologies involves deficits in the vasculo-neuronal-inflammatory triad, among others.

Current investigations will hopefully contribute to the development of highly specific drugs, such as the ideal anticoagulant or potent antiinflammatory and cytoprotective drugs. Modification of key structures in APC molecules is the basis to generate valuable information for drug engineering. This will aid in the engineering of a yet safer variant of APC devoid of increased bleeding risks, for use in cell-protection in the aforementioned relevant clinical conditions.

Acknowledgment

Funding sources: This work was supported by the PN de I+D+I 2008-20011 of Instituto de Salud Carlos III-Fondo de Investigación Sanitaria and FEDER (PS09/00610 and Red Temática de Investigación RECAVA RD06/0014/0004), Conselleria de Educación-Generalitat Valenciana (Prometeo/2011/027), and Fundación para la Investigación del Hospital Universitario La Fe (2007-0185), Spain. Pilar Medina is a Miguel Servet Researcher (ISCIII CP09/00065).

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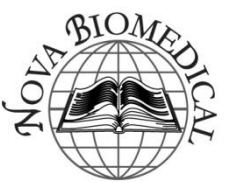
VENOUS THROMBOSIS

RISK FACTORS, MANAGEMENT AND COMPLICATIONS

CAROLINE H. GUTMANN
EDITOR



New York

Library of Congress Cataloging-in-Publication Data

ISBN: 978-1-63117-294-6 (eBook)

Library of Congress Control Number: 2014931439

Published by Nova Science Publishers, Inc. † New York

Contents

In: Venous Thrombosis
Editor: Caroline H. Gutmann
ISBN: 978-1-63117-293-9

Chapter 5

Current Knowledge of Genetic Risk Factors for Venous Thrombosis

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Abstract

Thrombophilias can be defined as a group of inherited or acquired disorders that increase the risk of developing thrombosis. Venous thromboembolism (VT) is considered a multifactorial disease produced by a sum of risk factors that predispose to the thrombotic event. This predisposition includes genetic and acquired defects. VT is one of the major public health problems worldwide, contributing to an estimated >500,000 deaths in Europe each year, with a mortality rate of 10% [1]. The risk of recurrence is about 6% per year, and post-thrombotic disease

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occurs within the next 5 years following a VT event in about 25% of patients [2, 3]. VT is a common multifactorial disease, with both established environmental and genetic risk factors [4].

The genetic basis of the VT is only partially known, and thrombophilia can only explain around 50% of the thrombotic events. Hence, the known risk factors, together with yet unknown alterations in any component of the haemostatic system, may allow us to further clarify the underlying mechanism of VT. The known genetic risk factors confer a poor relative VT risk, altogether can only explain about 5% of the VT heritability [5].

The identification of disease-associated VT genes suspected to be involved in the physiopathology of the disease could help to define its genetic determinants.

Moreover, the recent availability of high-throughput genotyping technologies and their application in the framework of genome-wide association studies (GWAS) have enabled the identification of novel susceptibility loci [6-12]. From 1965 to 2013, 16 genes/loci have been robustly associated with the susceptibility to VT [5], most of them affecting the coagulation cascade. In addition to the well-known established susceptibility genes for VT: *F5*, *F2*, *FGG*, *PROC*, *PROS*, *SERPINC1*, and ABO blood group, new emerging susceptibility loci have arisen: *C4BPA/C4BPB*, *F11*, *G6P*, *HIVEP1*, *KNG1*, *STXBP5*, *TCN2*, *VWF*. However, independent case-control studies are needed to confirm the association with the disease for these emerging genes. This chapter summarizes the current information on the role of genetic risk factors for venous thrombosis.

1. Well-Established Genetic Risk Factors for Venous Thrombosis

1.1. Factor V Gene

Dahlbäck et al. [13] described three families in whom activated protein C (APC) did not yield the expected prolongation of the clotting time in an activated partial thromboplastin time (aPTT) assay, and defined a new phenotype, called APC resistance (APCR). APCR co-segregates with the Factor V gene (*F5*), in particular with the FV Leiden mutation (g.1691G>T; rs6025), which encodes the substitution of arginine for glutamine at 506 position (R506Q), affecting one of the APC cleavage sites in the FV molecule [14]. This abnormal FV molecule is partially resistant to inactivation by APC, leading to a hypercoagulable state, and is the most common genetic risk factor

for familial venous thrombosis [14-16]. In Caucasians, the frequency of the FV Leiden mutation is around 5%, while in patients with VT is 21.0% [17, 18]. The risk of VT in heterozygous carriers of the FV Leiden mutation is approximately five fold higher than in the general population [19].

More recently, Smith et al. [20] identified the *F5* rs4524 variant, a non-synonymous substitution of lysine for arginine at the 858 residue (K858R), as a new VT-associated SNP. This SNP in *F5* was consistently associated with VT in three large Dutch case–control studies (MEGA-$_2$ OR=1.21; 95% CI=1.10-1.34; P<0.001), and linkage disequilibrium between this mutation and the FV Leiden was low ($r2$ = 0.02) [21]. In two French GWAS analyses, the OR for VT associated with the rs4524 allele was 1.17 (P = 0.013; PE Morange, DA Tregouet, unpublished data). The functionality of the rs4524 remains to be addressed.

When FV Leiden mutation is present in combination with other genetic risk factors, such as the presence of prothrombin 20210A mutation, haplotypes of the EPCR gene (*PROCR*) [22], or the ABO blood group [23], the risk of VT is modified, thus confirming the multifactorial origin of thrombosis.

1.2. Prothrombin Gene

Prothrombin (FII) has procoagulant, anticoagulant, and antifibrinolytic activities after its activation into thrombin by the prothrombinase complex. Thrombin acts by activating factors XIII, XI, VIII and V, as well as protein C (PC) and thrombin activatable fibrinolytic inhibitor. It also cleaves fibrinogen to form fibrin. So, the prothrombin gene (*F2*) was widely described as an obvious candidate associated with the risk of VT. It has been identified the prothrombin 20210A mutation (g.20210G>A; rs1799963) in the 3'-UTR region [24-26]. The prevalence of the prothrombin 20210A allele in the general population is 1.7-3.0%, while in VT patients is about 6.2% [18]. Carriers of the 20210A allele is associated with a 3-4 fold increased risk of VT [19], and increased prothrombin levels in plasma. This is probably due to a change in a polyadenylation efficiency of the protein [27], leading to increased thrombin generation, which results in a hypercoagulable state [24]. The mutation also contributes to the regulation of the PC anticoagulant pathway [28]. Carriership of the prothrombin 20210A allele, have an increased risk of recurrences [29], and the presence of other VT risk factors, such as the presence of the FV Leiden [30-34], *PROCR* haplotypes [35] or the ABO blood system [23], modifies their risk.

The mechanism by which prothrombin 20210A increases VT risk via increased prothrombin levels is not fully understood. There is some evidence that prothrombin levels affect the APC-resistance phenotype, but the precise mechanism is not known.

Another SNP located in the intron 13 of the *F2* gene (g.19911A>G; rs3136516), has been associated with increased prothrombin levels [36, 37]. An *in vitro* study [38] demonstrated that the rs3136516 is also functional through its effect on an intronic splicing enhancer motif [18, 25, 36-38].

1.3. Fibrinogen Gene

Fibrinogen is a key component of the haemostatic system, playing a role in both primary and secondary response [39]. Thrombin-catalyzed cleavage of fibrinopeptides A and B converts fibrinogen into fibrin, which spontaneously polymerizes, forming the fibrin clot. The fibrinogen is a disulfide-bonded-dimer molecule consisting of three polypeptides named Aα, Bβ, and γ. The three polypeptides are encoded by separate genes, *FGA*, *FGB*, and *FGG*, respectively, clustered in a region of approximately 50 kb [40]. The *FGG* mRNA transcript is subject to alternative processing and polyadenylation [41].

The main form, the γA chain, consists of 411 amino acids, and results from the use of the polyadenylation signal downstream of exon 10. Whereas, the alternative γ' chain results from the use of an alternative polyadenylation signal in intron 9.

Fibrinogen is a well-established predictor of cardiovascular disease outcomes such as myocardial infarction (MI) [42, 43] stroke, [44] and VT [45, 46]. It is estimated that individual variation in fibrinogen levels is heritable [47, 48], indicating a substantial influence of genetic factors. Several meta-analyses of fibrinogen GWAS studies, conducted in cohorts of European ancestry, identified several genetic variants affecting plasma fibrinogen levels [49, 50]. But, a recent meta-analysis of fibrinogen GWAS studies, based on a 4-fold greater sample size than other meta-analyses (>100000 subjects), identified up to 24 independent signals in 23 loci (of which 15 are new). Clinical outcome analysis of these loci does not support a causal relationship between circulating levels of fibrinogen and coronary artery disease, stroke, or VT [51].

Increased levels of fibrinogen have been proposed to promote fibrin formation, increase platelet aggregation and viscosity, or simply reflect an inflammatory state [52]. Several studies have highlighted the existence of a

first-order relationship between fibrinogen concentration and fibrinolysis rate [53], suggesting that increased fibrinogen levels may actually cause thrombosis by slowing fibrinolysis rates [54]. The association between fibrinogen γ′ and VT is controversial.

The first publication on this subject, a case-control study (N=948) from the Leiden Thrombophilia Study (LETS), showed that VT patients have reduced γA/γ' fibrinogen levels and reduced γ' fibrinogen/total fibrinogen ratio compared to healthy controls [46, 55]. There is a significant association between the *FGG*-H2 haplotype, that contains the *FGG* g.10034C>T SNP (rs2066865), and VT [46]. The *FGG*-H2 haplotype is associated with reduced γA/γ' fibrinogen levels and is robustly associated with an increased risk of VT [46]. Individuals with a γ' fibrinogen/total fibrinogen ratio below the 10^{th} percentile, as measured in the control group, had a 2.4-fold increased risk of VT in the LETS. A recent GWAS study demonstrated that the genetic loci associated with γ' fibrinogen levels are all located in or near the fibrinogen gene locus, the strongest association being observed with the *FGG* rs2066865 [56]. However this SNP is in linkage disequilibrium with the *FGA* Thr312Ala (rs6050) variation, that results in hard clots with increased Aα chain cross-linking, larger string diameters, and a lower number of string per unit area [57]. The authors suggested that γ′ fibrinogen was protective against VT due to its antithrombin (AT) activity [58]. It appears that the relationship between γA/γ' fibrinogen level and thrombosis may depend on the type of vascular disease, whereby the γ' chain associates with a prothrombotic risk in arterial disease, but with an antithrombotic effect in VT [46, 59]. It has been proposed that the γ′ chain has a high affinity binding site for active thrombin, and as a result, there is less free thrombin available to cleave its substrates. Therefore, low γ′ fibrinogen levels may cause reduced sequestration of thrombin, a hypercoagulable state and high risk of VT [60]. Binding of the γ′ chain to thrombin allosterically modulates the active site [61]. In addition, this AT activity also inhibits FVIII cleavage [62] and thrombin activation of platelets by limiting PAR1 cleavage [62, 63]. Studies with transgenic mice containing the human fibrinogen thrombin-binding γ′ chain sequence showed a decrease in thrombosis [64].

In contrast, Cheung et al. reported that γ′ fibrinogen levels during the acute phase of pulmonary embolism (PE) were highest [65]. Several studies have suggested that a possible explanation for these results is that, upon binding of thrombin to the γ chain, the cleavage of thrombin´s substrates is changed, such as thrombomodulin (TM) [66], platelet glycoprotein Ib α [67] or

AT [68]. This would potentially allow continued clot formation at the site where fibrin is being deposited, preventing diffusion of thrombin to blood flow and growing clot surface. Further studies will be necessary to definitively address the association between γ′ fibrinogen and VT.

1.4. Natural Anticoagulants of Coagulation and Risk of Thrombosis

The coagulation process is under the control of several inhibitors that limit clot formation near the damaged vessel wall, thus avoiding thrombus propagation. This delicate balance can be interrupted whenever the pro-coagulant activity of one of the coagulation factors is increased or the activity of one of the naturally occurring inhibitors decreases, leading to thrombus formation. Inherited AT, PC, and protein S (PS) deficiencies are rare but strong risk factors for venous thrombosis, although they have moderate or low effect on arterial thrombosis.

1.4.1. Antithrombin Gene

AT belongs to the serpin family of proteins and circulates in plasma acting as one of the most important natural anticoagulant that regulates coagulation by inhibiting procoagulant serine proteases such as thrombin [69, 70], activated (a) FX and FIX [71]. AT acts as suicide inhibitor, forming stable 1:1 enzyme-AT complexes in which the active site of the target proteinase, interacts with the reactive site of the inhibitor.

Based on the circulating antigen level and functional activity of AT, two types of AT deficiency can be distinguished. Type I AT deficiency is defined by a proportional decrease of the AT antigen and activity, whereas type II AT deficiency is characterized by a reduction of the AT activity and normal or almost normal AT antigen levels.

Decreased AT plasma levels have been associated with VT risk in a dose–response manner [72], suggesting that mutations which modulate AT levels variability could also be good candidates for VT risk. Egeberg was the first to identify a genetic risk factor for VT, the AT deficiency, by studying a family with many relatives affected by VT [73].

AT deficiency has been classified as a strong genetic risk factor for VT [74], with a relative thrombotic risk around 10 [19], although it is relatively rare in the general population (0.02%). AT deficiency is transmitted as an autosomal dominant trait, and due to the embryonic lethality of severe AT

deficiency, affected individuals are heterozygous [75, 76]. Since the original description [73], more than 227 distinct mutations in the AT gene (*SERPINC1*) have been described 216 of them related to AT deficiency [76, 77], approximately 50-60% of that mutations are missense [76, 78], and most of them reported an association between SNP and AT levels [79] and/or VT risk [12].

A GWAS study with ~900 individuals of the MARTHA project, identified several common SNPs (i.e. with minor allele frequency MAF > 5%) that causes a modified AT plasma levels [80]. However, there isn't any common SNP identified that could explain even 5% of plasma AT variability.

Homozygous AT deficiency is not compatible with life except for few homozygous or hemizygous patients with type II deficiency. Several AT deficiencies type II have been described. the most common being AT Budapest III (p.Leu131Phe) and AT Toyama (p.Arg79Cys) were associated with a soft thrombotic risk [81, 82], and AT Cambridge II (p.Ala416Ser), which is the most prevalent in normal population [83] causing an inefficient inhibition of thrombin [84], increase the potential thrombin generation [85], and a moderately increases the risk of venous and arterial thrombosis [83, 86].

Some family-based studies have evaluated the risk of thrombosis in AT deficient individuals and their relatives [87-89]. However, few studies have investigated the association between the risk of thrombosis in AT deficiency and *SERPINC1* mutations [83, 86, 90]. A recent retrospective cohort study showed that *SERPINC1* missense mutations are associated with a lower risk of VT and tended to have higher risk of arterial thrombosis compared to null mutations (deletions, insertions, nonsense mutations, and splice site mutations) [91].

1.4.2. Protein C Gene

PC is a vitamin k-dependent serine protease that acts as regulator of the coagulation cascade. PC circulates in plasma as a zymogen that is activated by the thrombin/TM complex [92], a reaction that is further enhanced by the endothelial PC receptor (EPCR) [93]. When activated PC (APC) is bound to its cofactor, the protein S (PS), it exerts an antithrombotic effect through proteolysis of the procoagulant FVa and FVIIIa, limiting the coagulation cascade and fibrin formation [94, 95]. Moreover, APC also exerts anti-inflammatory and cytoprotective activities, which includes antiapoptotic activity, neurological and endothelial barrier protection, and modulation of gene expression [96]. These activities require binding of APC to EPCR and

activation of the thrombin receptor PAR1 to initialize a cascade of cell signaling [97].

PC was described as a thrombotic risk factor in 1981, when Griffin et al. described a heterozygous PC deficiency in a family with a history of recurrent VT. The deficiency in homozygosis was described in 1984 by Seligsohn et al. [98] and Estellés et al. [92] in a newborn with disseminated intravascular coagulation and purpura fulminans within hours of birth.

There are two types of PC deficiencies: type I which is characterized by the reduction of antigen and functional levels, and type II, characterized by the presence of normal antigen levels and a reduction in the anticoagulant activity, which implies the presence of abnormal circulating PC molecules.

In the general population, low levels of circulating PC and APC is associated with an increased risk of VT [99, 100]. PC deficiency is usually due to privative mutations in the PC gene (*PROC*) and occurs in 1/ 250-500 of the adult population [19]. The thrombotic risk associated with heterozygous PC deficiency is approximately 8 fold, compared with individuals without any deficiency [19].

It is important to mention that the detection of both types of PC deficiencies is usually tested by measuring circulating PC levels and the functional activity of PC by chromogenic substrates. Thus, there are some undetected mutations that provide changes in substrate recognition sites, cofactor or membrane binding sites, which result in normal APC levels but reduced activity.

Several SNPs (including rs1799810, rs2069910, and rs2069915) located in the promoter of *PROC* mildly influence PC plasma levels [101-103], and also other SNPs located in other locus [104] (i.e. *PROCR and GCKR* loci [105]) will influence the plasma levels of PC.

Currently, there are more than 272 different mutations described in *PROC* and related to PC deficiency in the Human Gene Mutation Database (http:// www.hgmd.cf.ac.uk).

Several SNPs located in the promoter of the *PROC* gene have been shown to mildly influence the PC plasma levels [101-103]. A study in a Dutch population showed that the most frequent missense mutations were found in the Gln132 and Arg230 residues, leading to type I PC deficiency [106]. In French population, some studies showed that the most frequent type I mutations were in the Arg178 and Pro168 residues [107, 108]. Regarding type II PC deficiency, the change Arg to Gln in the residue 229, in a family with a history of VT [109].

Structure-function studies showed that the 229Gln mutation alters the activation of PC by the thrombin-TM complex [110, 111], which can explain the reduced biological activity of this PC variant, although PC plasma levels and enzymatic capacity remain unaltered.

1.4.3. Protein S Gene

PS is a 69 kD vitamin K-dependent plasma glycoprotein that acts as a non-enzymatic cofactor of APC and tissue factor pathway inhibitor [112-115].

PS circulates at a concentration of approximately 350 nM, which 40% is free and 60% forms inactive complexes with C4b-binding protein [116]. Only the free PS fraction has APC cofactor activity. PS deficiency is a risk factor for VT that affects approximately 5% of thrombophilic patients and 1–2% of consecutive patients with a first VT [16, 117]. Assessment of the thrombotic risk caused by PS deficiency is difficult due to the lack of sufficiently large studies examining its prevalence in the general population [118-123]. Although one large prospective case–control study failed to find an association between PS deficiency and venous thrombosis [118, 121], a 2.4 fold increased risk of thrombosis has been reported among PS deficient patients, while family-based studies have reported a 5–8.5 fold higher risk of thrombosis amongst affected relatives of PS deficient patients when compared with unaffected relatives [119, 120]. Three types of PS deficiency have been described: type I (decreased levels of both total and free PS antigen), type II (decreased APC cofactor activity but normal total and free PS antigen levels), and type III (decreased levels of free PS antigen levels only) [16, 117, 124]. Diagnosis of PS deficiency is complicated because of the inter- and intra-individual variation in plasma PS levels, and by the overlap in levels between unaffected individuals and those having heterozygous deficiencies. Several factors including age, sex, hormonal state, pregnancy, liver disease and inflammation can influence plasma PS levels, further complicating diagnosis [125-128]. The first VT patients with PS deficiency were described by Comp et al. in 1984 [129, 130]. Hereditary PS deficiency is due to rare and privative mutations within PS gene (*PROS1*). More than 234 mutations in *PROS1* have been described, the majority missense or nonsense mutations (http://www.hgmd.cf.ac.uk) [124, 131, 132], 210 of them related with PS deficiency. Sequencing of exons and splicing junctions of *PROS1* has lead to the identification of a mutation in approximately 50% of families with PS deficiency [131]. Moreover, large deletions and insertions in *PROS1* are present in approximately 30% of the point mutation-negative families [133-135]. Bertina et al. in 1990 [136] described the Heerlen polymorphism, which

causes a Ser460Pro substitution in the mature PS and is characterized by the presence of a PS molecule in plasma that has a lower molecular weight than normal PS. The biological significance of the Heerlen mutation is unclear. In one study, the allele was present in 1/192 (0.52%) chromosomes tested in a control population compared to 16/1182 (0.68%) chromosomes in consecutive VT patients [136]. However, in another study the Heerlen allele was shown to be more frequent among patients with type III PS deficiency than those with type I deficiency [137].

In addition, the Heerlen variant produces changes in the binding stoichiometry to C4BP-b that could result in a reduction in free PS levels.

In the MARTHA [80] and GAIT GWAS studies [138] common SNPs associated with PS levels were identified. These two studies failed to detect robust associations despite some suggestive elements in favor of an association of *DNAJC6* SNPs with free PS levels [138].

1.5. ABO Locus

High plasma levels of von Willebrand factor (vWF) and FVIII are well-established risk factor for VT [139]. Overall, approximately 70% of the variation in plasma levels of vWF and FVIII is genetically determined, with 30% explained by the individual ABO blood group [140]. Notably, vWF and FVIII levels are approximately 25% higher in individuals who have a non O-blood group [141-144]. The frequency of non-O blood group in patients with deep vein thrombosis (DVT) [143, 144] or VT [145-147] was significantly higher compared to the healthy blood donors [142]. Moreover, non-O individuals had higher concentrations of both factor VIII and VWF as compared to those group O subjects [142, 145, 146]. In addition, the risk was increased in non-O individuals carrying other inherited thrombophilic defects, such as FVL, prothrombin 20210A mutation, AT, PC and PS deficiencies [23, 142, 144, 146, 147].

Jick et al. [148] were the first to report that non-O blood group was associated with increased risk of VT. Recent studies [5, 8, 147, 149] have clarified this association and demonstrated that B and A1 blood groups had higher VT risk than O and A2 blood groups.

In addition, O and A2 groups can be genetically determined by the rs817 6719 and rs8176750 SNPs, and were consistently found at a lower frequency in VT patients than in controls in the three GWAS reported so far on VT [5, 8, 149, 150].

In depth analysis of the ABO locus, has recently identified several intronic and 3'UTR SNPs which evidenced that the association between VT and the ABO blood group could be independent of the rs8176750 and rs8176719 SNPs [149, 151]. All these studies emphasized the known complexity of the link between ABO locus and VT risk.

The mechanism by which ABO blood group is believed to contribute to VT risk is through modifications of VWF and FVIII levels in plasma, likely by affecting VWF glycosylation and thus its proteolysis and clearance [152, 153].

However, ABO blood groups remain significantly associated with VT even after adjustment for FVIII or vWF levels in cardiovascular diseases [146, 154].

This suggests that ABO may affect cardiovascular risk by other means than solely modifications of FVIII and vWF molecules.

First, although traditionally regarded as red blood cell antigen, ABO antigens are also expressed on various other tissues including platelets, the vascular endothelium, and epithelial surfaces [152].

Second, the ABO locus was found associated with plasma levels of soluble intercellular adhesion molecule-1, P-selectin, and E-selectin [155], three intermediate phenotypes of cardiovascular risk.

2. Emerging Genetic Risk Factors for Thrombosis

2.1. Von Willebrand Factor Gene

As previously mentioned, ABO blood group affects vWF glycosylation leading to increased VT risk [152, 153]. Moreover, genetic variation in the vWF gene (*VWF*) can also influence the risk of VT.

The *VWF* rs1063856 SNP, resulting in a Thr789Ala substitution in exon 18, is associated with increased vWF levels and VT risk (OR ~ 1.15) [156]. A meta-analysis of several GWAS datasets conducted under the CHARGE consortium [157] identified 6 novel loci modulating vWF plasma levels, CLEC4M, STX2, TC2N, STXBP5, SCARA5, and STAB 2 among which the latter three also influenced FVIII levels.

Therefore, these loci are natural candidates for VT risk. However none of the vWF-associated SNPs at the CLEC4M, STX2, and SCARA5 have been found so far associated with VT risk.

Conversely, TAB 2 [150, 158], TC2N [6], and STXBP5 [157] SNPs have been observed associated with VT risk but there is yet no evidence for a functional role of the STAB 2 and TC2N VT-associated SNPs.

The missense rs1039084 STXBP5 SNP (Asn436Ser) could be functional. Evidence for a role of STXBP5 in the regulation of vWF has been recently reinforced by the finding that STXBP5 SNPs were associated with a bleeding phenotype in women with type 1 von Willebrand disease [159].

2.2. Factor XI Gene

Meijers et al. [160] reported that high FXI levels were associated with increased risk of VT. FXI circulates as a zymogen as a complex with high molecular weight kininogen (HMWK) [161] and contributes to haemostasis by activating FIX. FXI can be activated by four biologically relevant proteases: FXIIa, FXIa, thrombin, and meizothrombin. The most relevant *in vivo* pathway for plasma FXI activation seems to be the feedback activation by thrombin [162]. Later, in 2007, several SNPs, including rs2036914, rs3822057 and rs2289252, at the *F11* locus were reported to be associated with the risk of VT (with OR ~1.35) through modulation of FXI plasma levels [12, 20, 163].

2.3. Glicoprotein 6 Gene

The glycoprotein 6 gene (*GP6*) is the first VT-associated locus that lies outside the standard coagulation cascade. It was identified through a large-scale association study, focusing mainly on non-synonymous polymorphisms [12]. *GP6* encodes the receptor glycoprotein VI that has a major role in collagen induced signalling.

It is a crucial platelet membrane glycoprotein, for adequate platelet activation, adhesion and aggregation. The *G6P* rs1613662 (Ser219Pro) SNP, was associated with a 15% increased risk of VT [8, 164]. Although the precise mechanism is unknown, the Ser219Pro change modified the collagen receptor GPVI, and produced a reduction in the collagen-induced platelet activation in a dose-dependent manner [165]. The identification of *GP6* as a VT-associated locus is striking as it indicates that platelets play a determining role in VT although they have historically been ignored in this pathological setting. Activated platelets are important catalysts of both intrinsic and extrinsic thrombin generation and, therefore, fibrin deposition [166]. Kotuličová D et al.

2012 studied the genetic variability of *GP6* in patients with platelet hyperaggregability phenotype, known as sticky platelet syndrome (SPS) and manifesting as DVT, and/or pulmonary embolism. A significant association between 1613662-G [P < 0.05, odds ratio (OR) 2.087, confidence interval (CI) 1.049-4.148], and VTE was found in patients with SPS. They also found association between VTE and another GP6 rs1654419 SNP (P<0.05, OR=2.161, CI 95%=1.020-4.577), in patients with SPS. However, there was not found association between the studied GP6 genotypes and the severity of VTE (pulmonary embolism vs. DVT) was found [167].

2.4. Protein C System and Risk of Thrombosis

As previously mentioned the PC system plays a crucial role in the regulation of blood coagulation in humans.

APC generation depends on the precise coupling, on the surface of endothelial cells, of thrombin and PC to their respective receptors, TM and EPCR. Any change in the efficiency of this coupling may cause altered APC generation and a modification in the risk of thrombosis. In fact, several mutations have been reported in *PROCR* or TM gene (*THBD*), some of them associated with the risk of thrombosis.

2.4.1. EPCR Gene

EPCR is a 46-kD type I transmembrane protein, constitutively expressed on the luminal surface of the endothelial cells of large vessels, and is structurally similar to the major histocompatibility class 1/CD1 family proteins involved in the immune and inflammation responses [168]. Thus, it expression had been reduced by inflammatory stimuli, like tumor necrosis factor-α, or an atherosclerotic setting [168, 169]. EPCR is also expressed in the surface of other cell types [170-182], which suggests the role of EPCR in other disorders. In fact, Animal experiments have demonstrated the importance of EPCR in normal embryonic development, in *PROCR* knockout mice, fibrin deposition in trophoblast giant cells results in thrombosis at the maternal-embryonic interface [183].

A soluble form of EPCR (sEPCR) also circulates in the plasma. By limiting APC generation and function, elevated levels of sEPCR may exert procoagulant and proinflammatory effects; in 2 case-control studies [35, 184] elevated levels of sEPCR were associated with increased risk of VT. Likewise,

a small family study found a higher occurrence of VT in those with above normal values of sEPCR compared with those with normal levels [185].

Functional polymorphisms in the *PROCR* gene may increase/decrease the risk of thrombosis, especially in carriers of other prothrombotic mutations, as VT is a multifactorial disease [186, 187]. Rare point mutations within the gene [188] and its promoter region [189] have been described, but their effect on thrombosis and gene expression remain unknown given its low population frequency [190]. The first *PROCR* gene mutation described was a 23bp insertion in exon 3, at position 4031 [191], and results in a STOP codon downstream from the insertion point [188]. Expression studies in mammalian cells showed that the truncated protein is not localized on the cell surface, can not be secreted in the culture medium, and does not bind APC, suggesting that the insertion is a risk factor for arterial and venous thrombosis. However, given its low population frequency (<1%) [192, 193], statistical analysis did not reveal a significant association between the mutation and the risk of thrombosis [188, 191, 192, 194-196].

Franchi et al. [197] described a mutation in the promoter region of the *PROCR* gene, g.(-318)T>G substitution, present in 1/95 women with late fetal loss and in 0/236 controls. Biguzzi et al. [189] reported four point mutations in the *PROCR* gene promoter.

However, they were rare in patients with VT or MI, and the *in vitro* characterization did not reveal any decreased activity of the reporter gene in basal conditions. Hermida et al. identified a heterozygous C to T variation at position 2769, which predicts a substitution of Arg to Cys at position 98 in the mature protein [198]. However, its low population prevalence and the *in vitro* expression and characterization of this mutation suggested no role of this mutation in VT [198].

Simmonds and Lane [199] reported the organization and nucleotide sequence of the human *PROCR* and suggested two potential polymorphic sites, the g.2532T>C substitution in intron 1 and the g.4600A>G substitution in exon 4 that predicts a Ser219Gly change.

In the last years, up to four haplotypes of *PROCR* have been reported [184, 200, 201]: H1, H2, H3 and H4, three of which contain one or more haplotype-specific SNPs, while H2 contains the common allele of each SNP.

The *PROCR* H1 haplotype tagged by the rare allele of g.4678G>C (rs9574), has been associated with increased circulating APC levels [202] and a reduced risk of VT in 2 independent studies (OR = 0.59, 95% CI=0.41–0.84) [201, 203]. H1 also reduced the risk of thrombosis in carriers of FV Leiden (OR=0.31; 95%CI=0.16–0.83) [22].

In patients with the FV Leiden mutation, the mean age at the first thrombosis was significantly higher in propositi carrying the H1H1 genotype than in non-carriers of the H1 haplotype. In addition, the probability of being free of thrombosis at age 40 was significantly higher in propositi carriers of the H1H1 genotype than in the non carriers ($P<0.001$). In contrast, two other groups found no association of the H1 haplotype with the risk of thrombosis [200] or a slight reduction of the risk [184].

A possible explanation for the protective effect of the *PROCR* H1 haplotype against VT, both in patients with no thrombophilic mutations and in carriers of FV Leiden, would be its association with increased APC levels. It has been described that a low level of APC in plasma is a strong, prevalent, independent risk factor for VT [100, 204].

In fact, reduced APC levels increased the risk of VT 4.7-fold, and a preliminary familial study revealed that in some instances the low APC phenotype is hereditary.

Presently it is unknown which SNP in EPCR H1 is responsible for the reported protective effect.

The H1 haplotype contains 10 specific alleles, the 1451T (rs2069943), 1541A (rs2069944), 1880C (rs2069945), 2532C (rs2069948), 2897A (rs945 960), 3424C (rs871480), 3997C (rs2069952), 4678C (rs9574), 5632G (rs1415 773), and 5663A (rs1415774). Therefore, any of these nucleotides may be responsible for the observed association of the *PROCR* H1 haplotype with increased levels of plasma APC and reduced risk of VT. Further studies are required to identify which polymorphism/s is/are responsible for the observed associations. The *PROCR* H3 haplotype tagged by the rare allele of g.4600A>G (rs867186), is associated with increased plasma levels of sEPCR [35, 184, 200, 201, 205-208] and increase VT risk [105, 200, 209].

Regarding the SNPs that comprise the *PROCR* H3 haplotype, the 4600G allele is the more obvious candidate responsible for the association of *PROCR* H3 haplotype with increased sEPCR levels, in view of the fact that the cleavage of the EPCR anchored in the endothelial cell membrane to generate sEPCR occurs within the region of the protein encoded by exon 4, near the 4600 position. The 4600G variant predicts a conformational change in the protein due to the Ser 219 to Gly substitution, which could render an EPCR more susceptible to cleavage by metalloprotease such as ADAM17 [200, 201]. This hypothesis has been supported by two independent studies [206, 210].

Other mechanism that could link the H3 haplotype and high levels of sEPCR is the alternative splicing reported by Saposnik et al. [211]. In both cases, the increased shedding of EPCR could reduce the amount of EPCR at

the endothelial surface and explain the increased Vt risk associated with H3. In favor of this argument is the fact that inducing EPCR shedding in cells bearing the H3 haplotype notably reduced their hability to sustain PC activation as compared with non-H3 cells [210].

Studies looking for genetic determinants for PC levels have shown that the *PROCR* H3 is associated with higher plasma PC levels [103, 105]. Additionally, the H3 haplotype has also been associated with higher levels of FVII [157, 212], which could also explain the increased VT risk associated to this haplotype. One study demonstrated that *PROCR* H3 also increases the risk of VT in carriers of the prothrombin 20210A mutation, probably due to its association with increased sEPCR levels, and that H3 carriers experienced the first VT episode at a young age [35].

Nevertheless, the molecular mechanism by which the increased sEPCR levels observed in individuals bearing the H3 haplotype could increase the risk of venous thrombosis remains to be determined.

It has been described that an increased physiological sEPCR concentration result in decreased PC activation and inhibition of generated APC [200].

Finally, *PROCR* H4 haplotype was reported to be associated with a slight increase in the risk of VT [184], although no further studies have confirmed these results.

2.4.2. Thrombomodulin Gene

TM is an endothelial cell membrane protein that acts as a receptor for thrombin in the activation of PC. TM is also independently involved in cytoprotective responses [213-216]. Studies in animal models suggest that TM deficiency is associated with a prethrombotic state [217-219].

Heterogeneous soluble forms of TM (sTM) circulate in plasma [220] and their levels are increased in several clinical conditions [221]. Some of these soluble forms might have anticoagulant activity [222]. According to the assay used for the measurements, range from 2.7 to 5.4 ng/ml [223] or from 25 to 65 ng/ml [224].

Genetic studies have identified a number of mutations and polymorphisms in the *THBD* gene, but no clear association with venous or arterial thrombosis has been found [225]. A common SNP in the coding region of *THBD* (c.1418 C>T; rs1042579), which results in the replacement of Ala455 by Val, has been described [226]. This dimorphism is located in the TM region responsible for thrombin binding and PC activation, suggesting a potential role in the modulation of TM function.

However, its association with venous [226-230] and arterial thrombosis [231-233] is not consistent.

Several studies have investigated the association between the *THBD* c.14 18C>T polymorphism and venous and arterial thrombosis, with conflicting results. Some studies did not find an association between the polymorphism and the risk of VT [229, 230]. Others have described a lower risk of VT in blacks carrying the *THBD* 1418T allele [229] or a protective effect for the T allele in arterial thrombosis [232], whereas others reported a higher risk or VT in the presence of the *THBD* 1418T allele in Japanese population [234] or an increased risk of coronary heart disease associated with the presence of the *THBD* 1418T allele in Caucasians [235] and black population [233].

An explanation for these discrepancies may lie in differences in study designs and patient populations, age and small sample sizes.

A recent large case-control study [236], in Caucasians, provided new data on the age dependent association between the presence of the *THBD* 1418T allele and a lower VT risk.

Moreover, additional analysis in this study, showed that human umbilical vein endothelial cells (HUVEC) carrying the *THBD* 1418T allele (Val455), had lower sTM levels in culture supernatant, increased membrane–bound TM, and increased cellular TM activity, defined as the thrombin-mediated PC activation on intact HUVEC, whereas the *THBD* mRNA level was similar in carriers and non-carriers. The mechanism by which the *THBD* 1418T allele protects against VT is not clear.

One explanation could be that the Val455 form has higher cofactor activity for PC activation. However, recombinant Val455 and Ala455, produced by Cos-1 cells, were found equally active in PC activation [237]. Another explanation could be that the *THBD* 1418T allele is associated with increased membrane-bound TM, resulting in increased PC activation, but no significant differences in *THBD* mRNA levels according to the *THBD* c.1418 C>T polymorphism in HUVEC cell studies were found [236]. Soluble TM represents cleaved forms of membrane-bound TM with loss of part of the serine–threonine rich region, the transmembrane domain, and the cytoplasmic tail [238], and the TM 455Ala residue is located not far from the presumed cleavage site.

So it is possible that the membrane-bound TM carrying the 455Val (1418T allele) might be more stable and less prone to shedding and may induce a protection from TM cleavage by proteases, which will result in higher PC activation rate and a lower risk of VT. Regarding to the levels of sTM, the association is controversial.

It has been reported that increased levels of sTM are associated with higher VT risk, and this association was, at least in part, independent of the *THBD* c.1418C>T polymorphism [236].

In contrast, increased sTM levels have also been reported in patients with recurrent VT [239], and other studies did not find any association between sTM and risk of VT [229].

Alternatively, the observed association between the *THBD* c.1418C>T polymorphism and the risk of VT could be attributed to another polymorphism in tight linkage disequilibrium with c.1418C>T.

In fact, some studies observed complete linkage disequilibrium ($r2$=0.98) between the c.1418C>T and c.2729 A>C (rs3176123) polymorphisms, and a moderate linkage disequilibrium between the c.1418C>T and c.–1208-1209del TT ($r2$=0.27) [236, 240].

2.5. Emerging Genes

A new estimate of the genetic variance associated with VT susceptibility was also derived using several GWAS and meta-analysis [6-12], conducted on thousands of individuals.

These emerging genes were robustly associated with VT, although, further analyses in case-control studies are necessary to confirm their association.

Results from GWAS and gene expression analyses [11] identified the *C4 BPA/C4BPB* genes as new susceptibility loci for VT with unknown PS-independent mechanism. *C4BPA* and *C4BPB* genes coding for the α- and β-chains of the C4b-BP, a heterodimeric molecule existing in three different isoforms: α7β1, α6β1, and α7β0. The rs3813948-C allele was found associated with increased levels of both α7β0 and *C4BPA* expression and increased risk of VT in two French case–control studies [11].

This SNP was not associated with PS levels, this emphasizes the previously raised possibility that C4b-BP can be independently active in the coagulation pathway [241].

Moreover, C4b-BP also belongs to complement inactivate proteins which are thought to be involved in immune response and inflammation, and this could favor a role of inflammation in VT-related mechanisms [242]. Another association was observed for the *SERPINF2* rs8074026. *SERPINF2* is an obvious candidate for VT as it codes for a serpine protease inhibitor that acts as plasmin inhibitor.

However, no trend for association was observed in the replication study. Two GWAS analyses identified the *STAB2* rs1593812 / rs4981021 haplo-type to increase the risk of VT [158]. *STAB2* encodes for stabilin-2.

HIVEP1 encodes a protein that participates in the transcriptional regulation of inflammatory target genes. A GWAS analyses showed that *HIV EP1* rs169713 SNP increase VT risk [10] and the rs169715/ rs2228220 haplo-type (r2 = 0.09) also increase the VT risk, but with an additive effect [150]. Following the findings of a GWAS on aPTT levels [243], a candidate bio-marker for VT it has been recently suggested, the *KNG1* gene, encoding the HK protein. HK plays an important role in blood coagulation bringing near prekallikrein, FXI and FXII [244].

Later, another study [245], showed that the *KNG1* rs710446 (p.Ile581Thr) SNP was also associated with increased VT.

More recently, the GAIT study [246] has shown that the rs710446 SNP was also associated with plasma FXI concentration.

3. Final Remarks

This chapter summarizes the main current information on the role of genetic risk factors for venous thrombosis. Pathogenesis of thrombosis has to be considered within a multifaceted perspective, as confirmed by the amount of epidemiological data on both genetic and environmental thrombotic risk factors.

Nevertheless, a significant proportion of thrombotic episodes, especially among young individuals, occur without a plausible explanation. On the other hand, many thrombosis patients have more than one predisposing genetic and/ or environmental risk factor, and there is convincing evidence that risk increases in proportion to the number of predisposing factors.

Traditional thrombotic risk factors, environmentals, acquired and genetics, play a role in VT, but fail to fully explain the pathology. Further basic and clinical research is needed to reach a correct identification of new factors associated with VT and/or arterial thrombosis, in order to assess the individual risk of thrombosis and promote more targeted prophylactic and therapeutic options.

Acknowledgments

Funding sources: This work was supported by the PN de I+D+I 2008-20011 of Instituto de Salud Carlos III-Fondo de Investigación Sanitaria and FEDER (PI12/00027 and Red Temática de Investigación RIC RD12/0042/0029), Conselleria de Educación-Generalitat Valenciana (Prometeo/2011/027), and Fundación para la Investigación del Hospital Universitario La Fe (2007-0185), Spain. Pilar Medina is a Miguel Servet Researcher (ISCIII CP09/000 65).

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