UNIVERSIDAD DE COSTA RICA SISTEMA DE ESTUDIOS DE POSGRADO

ALTERACIONES GENERADAS POR DIFERENTES TIPOS DE METALOPROTEINASAS HEMORRÁGICAS DE VENENOS DE SERPIENTES SOBRE PROTEÍNAS DE LA MEMBRANA BASAL Y OTROS COMPONENTES DE LA MICROVASCULATURA

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado del Doctorado en Ciencias para optar al grado y título de Doctorado Académico en Ciencias

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DEDICATORIA

A mi familia, especialmente a mis padres, porque gracias a ellos he logrado alcanzar mis metas, y a mi esposo Juan por su apoyo incondicional durante todo este proceso

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RESUMEN

Las metaloproteinasas de venenos de serpientes (MPVSs) son las toxinas responsables de la hemorragia local y sistémica en el envenenamiento por especies de la familia Viperidae. Se ha demostrado que estas toxinas actúan principalmente sobre la microvasculatura al degradar componentes de la membrana basal (MB) y otras proteínas de la matriz extracelular (MEC), lo que provoca un debilitamiento del capilar, seguido de ruptura y extravasación de componentes sanguíneos. Sin embargo, aún no se ha logrado identificar los componentes sobre los que actúan estas toxinas y si existen diferencias entre los tipos de MPVSs que permitan explicar las variaciones en la actividad hemorrágica entre ellas. Además, existe la necesidad de contar con modelos in vivo que permitan estudiar los efectos de las MPVSs sobre los diferentes componentes de la microvasculatura. Por lo tanto, en el presente trabajo se estudió el patrón de distribución y la capacidad de unión a componentes de la MB vascular de las MPVSs hemorrágicas: BaP1 (PI), BlatH1 (PII) y CsH1 (PIII), y una MPVS no hemorrágica: la Basparina (PIII), por medio de pruebas de inmunofluorescencia y microscopía confocal en músculo cremáster murino. Además, en este mismo modelo se comparó las alteraciones inducidas por cantidades equi-hemorrágicas de las MPVSs BaP1 y CsH1 sobre los diferentes componentes de la microvasculatura (i.e. células endoteliales, MB y células de músculo liso/pericitos), en los diferentes tipos de vasos (i.e. arteriolas, capilares y vénulas post-capilares-VPC) y en condiciones con y sin flujo sanguíneo. También se identificó las principales proteínas de la MB y MEC que son degradadas in vivo por cantidades equi-hemorrágicas de las MPVSs BaP1, BlatH1 y CsH1 y por el veneno completo de B. asper, por medio de pruebas inmunoquímicas y estudios de proteómica en modelos murinos que utilizan homogenizado de piel y exudado inflamatorio obtenido del músculo gastronemio. Los resultados muestran que las MPVSs hemorrágicas tipo PII y PIII se unen a componentes de la MB vascular y colocalizan con el colágeno IV, mientras que la MPVS no hemorrágica tipo PIII no se une a componentes de la MB y la MPVS hemorrágica tipo PI se distribuye de manera más difusa en el tejido. Esta distribución difusa de la BaP1 puede estar asociada con los efectos adicionales que se observaron para esta MPVS, como relocalización de la VE cadherina en VPC con aumento de la permeabilidad vascular, y aumento del tamaño de las hendiduras entre las células de músculo liso y pericitos en arteriolas y VPC; además, esta MPVS mostró una acción proteolítica generalizada sobre una mayor cantidad de componentes de la MEC en comparación a las MPVSs tipo PII y PIII. Por otro lado, se demostró que las MPVSs BaP1 y CsH1 son capaces de degradar el colágeno IV de la MB vascular, tanto en presencia como en ausencia de flujo sanguíneo, siendo más susceptible la MB de capilares; además, las tres MPVSs hemorrágicas generaron patrones de degradación similares para el colágeno IV presente en el homogenizado de piel y en el exudado inflamatorio. También se encontró que los productos de degradación del colágeno IV, perlecan y colágenos no fibrilares aparecen en el exudado inflamatorio en mayor cantidad en la primera hora del envenenamiento, debido a la rápida acción de las MPVSs, y luego su cantidad disminuye con el tiempo. Además, el perlecan aparece en cantidades importantes y similares en el exudado inflamatorio inducido por las tres MPVSs. Estos hallazgos sugieren que el colágeno IV, y posiblemente el perlecan, pueden ser blancos claves de las MPVSs en el mecanismo mediante el cual estas toxinas inducen hemorragia. Palabras claves: metaloproteinasas, venenos de serpiente, hemorragia, membrana basal, matriz extracelular, microvasculatura

ABSTRACT

Snake venom metalloproteinases (SVMPs) are the main toxins responsible for local and systemic hemorrhage in envenomings by species of the family Viperidae. It has been demonstrated that these toxins act mainly on the microvasculature by degrading components of the basement membrane (BM) and other extracellular matrix (ECM) components, which causes a weakening of capillaries, followed by rupture and extravasation of blood components. However, it has not yet been possible to identify the components on which they act, and whether there are differences between the types of SVMPs that could explain their variable hemorrhagic activities. In addition, in vivo models are required to assess the effects of SVMPs on different components of the microvasculature. Therefore, in the present work we studied the distribution pattern and binding capacity to components of the vascular BM of the hemorrhagic SVMPs: BaP1 (PI), BlatH1 (PII) and CsH1 (PIII), and a nonhemorrhagic SVMP: Basparin (PIII), by immunofluorescence and confocal microscopy in murine cremaster muscle. In addition, we used this model to compare the alterations induced by equi-hemorrhagic amounts of BaP1 and CsH1 SVMPs on the different components of the microvasculature (i.e. endothelial cells, BM and smooth muscle cells/pericytes), in the different types of vessels (i.e. arterioles, capillaries and post-capillary venules-PCV), and in conditions with and without blood flow. We also identified the main proteins from the BM and ECM degraded in vivo by equi-hemorrhagic amounts of BaP1, BlatH1 and CsH1 SVMPs, and by B. asper venom, in skin homogenates and inflammatory exudate collected from gastrocnemius muscle in mice by immunochemical tests and proteomics studies. The results show that hemorrhagic PII and PIII SVMPs bind to components of vascular BM and colocalize with type IV collagen, while the non-hemorrhagic PIII SVMP does not bind to components of the BM, and hemorrhagic PI SVMP is distributed more diffusely in the tissue. This diffuse distribution of BaP1 may be associated with the additional effects observed for this SVMP, such as relocation of the VE cadherin in PCV with an increase in vascular permeability, and an increase in the gaps size between smooth muscle cells and pericytes in arterioles and PCV; in addition, it induced a more widespread proteolytic action on components of the ECM as compared to PII and PIII SVMPs. On the other hand, we demonstrated that BaP1 and CsH1 SVMPs are able to degrade type IV collagen from the vascular BM, both in presence and absence of blood flow, with a higher susceptibility of the BM of capillaries. Moreover, the three hemorrhagic SVMPs generated similar degradation patterns for type IV collagen from skin homogenate and inflammatory exudate. In addition, degradation products of type IV collagen, perlecan and non-fibrillar collagens were detected in the inflammatory exudate in greater quantity in the first hour of the envenoming, highlighting the rapid action of SVMPs present in the venom, and then their amount decreases over time. On the other hand, perlecan appears in greater and equal quantities in the inflammatory exudate induced by the three SVMPs. These findings suggest that type IV collagen, and probably perlecan, may be key targets of SVMPs in the mechanism of hemorrhage induced by these toxins.

Keywords: metalloproteinases, snake venom, hemorrhage, basement membrane, extracellular matrix, microvasculature

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ratón

LISTA DE ABREVIATURAS

ADAMs:	Enzimas con un dominio "tipo disintegrina", un dominio rico en
	cisteína y un dominio metaloproteinasa
ADAMTs:	Enzimas con un dominio "tipo disintegrina", un dominio rico en
	cisteína, un dominio metaloproteinasa y motivos trombospondina tipo I
CM:	Carboximetil
DEAE:	Dietilaminoetil
DC:	Dominios tipo disintegrina y rico en cisteína
EEM:	Error Estándar de la Muestra
EDTA:	Ácido dietilamino tetraacético
FACITS:	Colágenos asociados a los fibrilares con triple hélice interrumpida
FPLC:	Cromatografía líquida rápida de proteínas
GAPDH:	Gliceraldehído-3-fosfato deshidrogenasa
LC/MS/MS:	Cromatografía líquida masas/masas
LRP5/6:	Proteína relacionada al receptor de lipoproteína de baja densidad 5 y 6
MB:	Membrana basal
MEC:	Matriz extracelular
MM:	Masa molecular
MMPs:	Metaloproteinasas de matriz extracelular
MPVSs:	Metaloproteinasas de venenos de serpiente
MS:	Espectrometría de masas
PBS:	Solución amortiguadora de fosfatos
PLA ₂	Fosfolipasa A2
SDS:	Dodecilsulfato sódico
SDS-PAGE:	Electroforesis en gel de poliacrilamida con dodecilsulfato sódico
αSMA:	Actina α del músculo liso
VE cadherina:	Cadherina vascular endotelial
VPC:	Vénulas post-capilares
vWF:	Factor von Willebrand

LISTA DE PUBLICACIONES

Esta Tesis se basa en los siguientes artículos:

- I. <u>Herrera C</u>, Escalante T, Voisin MB, Rucavado A, Morazán D, *et al.* Tissue localization and extracellular matrix degradation by PI, PII and PIII snake venom metalloproteinases: clues on the mechanisms of venom-induced hemorrhage. PLOS Neglected Tropical Diseases 2015; 9(4): e0003731.
- Herrera C. T, Voisin MB, Escalante T, Rucavado A, Nourshargh S, Gutiérrez JM. Effects of PI and PIII snake venom haemorrhagic metalloproteinases on the microvasculature: a confocal microscopy study on the mouse cremaster muscle. PLoS One 2016; 11(12): e0168643.
- III. <u>Herrera C</u>, Macêdo JKA, Feoli A, Escalante T, Rucavado A, Gutiérrez JM, Fox JW. Muscle tissue damage induced by the venom of *Bothrops asper*: identification of early and late pathological events through proteomic analysis. PLOS Neglected Tropical Diseases 2016; 10(4): e0004599.
- IV. Gutiérrez JM, Escalante T, Rucavado A, <u>Herrera C</u>, Fox JW. A comprehensive view of the structural and functional alterations of extracellular matrix by Snake Venom Metalloproteinase (SVMPs): novel perspectives on the pathophysiology of envenoming. Toxins 2016, 8, 304; doi:10.3390.

1. INTRODUCCIÓN

1.1. Venenos de serpiente de la familia Viperidae

El accidente ofídico constituye un importante problema de salud pública a nivel mundial, principalmente en los países tropicales y subtropicales, donde además es considerado un problema de salud ocupacional ya que la población más afectada son hombres jóvenes agricultores [1–4]. La Organización Mundial de la Salud estima un total de 2,5 millones de casos anuales de envenenamientos por mordeduras de serpientes con 125000 muertes en todo el mundo. En América Latina se estiman 150000 casos de envenenamientos y 5000 casos de muertes anuales [1].

En América Latina, la mayoría de estos accidentes ofídicos son causados por especies de la familia Viperidae. A esta familia pertenecen géneros como *Crotalus, Bothriechis y Bothrops*, entre otros. Los venenos de estas serpientes contienen varias proteínas biológicamente activas que alteran diversos procesos fisiológicos. Los mayores componentes de estos venenos son las metaloproteinasas de venenos de serpiente (MPVSs), las fosfolipasas A₂ (PLA₂) y las serina proteínasas. Otros componentes en menor proporción son disintegrinas, proteínas de la familia de las lectinas tipo C, L-amino ácido oxidasas, hialuronidasas y péptidos potenciadores de bradiquininas [5–7].

El cuadro clínico que se desarrolla tras el envenenamiento por las serpientes de esta familia se caracteriza comúnmente por la presencia de efectos locales como dolor, edema, hemorragia y mionecrosis en el sitio de la mordedura y efectos sistémicos como hemorragia, coagulopatías, alteraciones renales y choque cardiovascular [4,6].

El dolor y el edema local se generan por una serie de factores, entre ellos la acción directa de las MPVSs sobre la microvasculatura, así como por la respuesta de los tejidos a la acción del veneno, lo que provoca la síntesis y liberación de mediadores endógenos de la inflamación [8]. La mionecrosis se debe principalmente a la acción de las PLA₂, las cuales interactúan específicamente con la membrana plasmática del músculo esquelético, desencadenando una serie de eventos que culminan en un daño celular irreversible [9,10].

Las alteraciones de la hemostasia que se producen tras el envenenamiento por estas serpientes están asociadas a cuadros de desfibrinación, coagulación intravascular diseminada y trombocitopenia. Estos efectos se deben a la acción de diferentes proteínas como son serina proteinasas "tipo trombina" que transforman el fibrinógeno en fibrina, MPVSs que activan los factores de la coagulación II y X [11] y disintegrinas y proteínas de la familia de las lectinas tipo C que alteran la agregación plaquetaria [6,12].

La hemorragia local y sistémica es uno de los efectos más comunes en el envenenamiento y se produce principalmente por acción de las MPVSs [13,14]. La hemorragia causada por estas toxinas contribuye a efectos locales como la necrosis muscular debido a la isquemia [8,9] y complicaciones sistémicas como hipovolemia, hipotensión, choque cardiovascular, accidentes cerebrovasculares y daño renal [15]. Los antivenenos, o sueros antiofídicos, son muy eficaces en la neutralización de la hemorragia sistémica inducida por los venenos; sin embargo, muchas veces la neutralización de los efectos locales se logra sólo parcialmente, debido a la rapidez con que se desencadenan y al retardo en la administración del antiveneno, lo que puede dejar lesiones incapacitantes en el sitio de la mordedura [2,4]. Por lo tanto, el estudio de estas toxinas es importante, no solo para ampliar el conocimiento de la patogénesis del envenenamiento ofídico, sino también para el desarrollo de futuros antivenenos y tratamientos más efectivos que prevengan o minimicen los efectos locales y sistémicos debidos a la hemorragia.

1.2. Metaloproteinasas de venenos de serpientes

Las MPVSs son enzimas proteolíticas sintetizadas en la glándula de las serpientes en forma de zimógenos y cuya actividad catalítica es dependiente de zinc [16,17]. Las MPVSs se clasifican estructuralmente dentro de la subfamilia de metaloproteinasas denominada adamalisinas o M12B. A esta subfamilia también pretenecen las metaloproteinasas de mamíferos denominadas ADAMs, enzimas que poseen un dominio "tipo disintegrina" y otro rico en cisteína, además del dominio metaloproteinasa, y las ADAMTs, enzimas que poseen, además de los dominios anteriores, un dominio con motivos trombospondina tipo I. A su vez, las adamalisinas forman parte de la familia de metaloproteinasas dependientes de zinc

denominadas metzincins o M12. A esta familia también pretenecen las metaloproteinasas de matriz extracelular (MMPs, por sus siglas en inglés), entre otras [14,18–20].

Por otro lado, las MPVSs se clasifican en tres grupos desde el punto de vista estructural dependiendo de la presencia de ciertos dominios: 1) PI, con una masa molecular entre 20 y 30 kDa, presentan únicamente el dominio metaloproteinasa; 2) PII, con una masa molecular entre 30 y 60 kDa, presentan el dominio metaloproteinasa seguido de un dominio disintegrina, el cual muchas veces es liberado por acción proteolítica; 3) PIII, con una masa molecular entre 50 y 90 kDa, contienen un dominio metaloproteinasa, un dominio "tipo disintegrina" y un dominio rico en residuos de cisteína. A este último grupo pertenecen también un grupo de MPVSs que contienen adicionalmente una subunidad similar a lectina tipo-C unida por puente disulfuro [14,21]. En cada uno de estos grupos existen subgrupos que presentan variaciones estructurales dentro del esquema básico mencionado.

La actividad hemorrágica de estas toxinas se ha asociado con la actividad proteolítica, debido a que agentes quelantes del zinc eliminan los efectos proteolíticos y hemorrágicos [22–26]. Sin embargo, existen MPVSs de tipo PIII, que carecen de actividad hemorrágica, pero poseen efectos coagulantes al ser activadores de los factores de la coagulación II y X [27,28]. Tal es el caso de la Basparina del veneno de *Bothrops asper*, la cual es una MPVSs de tipo PIII activadora de protrombina que carece de actividad hemorrágica [27]. También existen MPVSs de tipo PI que poseen actividad proteolítica pero no actividad hemorrágica [29,30].

Por otro lado, existen diferencias en la actividad hemorrágica de los diferentes tipos de MPVSs. En general, las MPVSs de tipo PIII poseen una mayor actividad hemorrágica con efectos tanto locales como sistémicos [24,31]; mientras que las MPVS de tipo PI poseen una menor potencia hemorrágica con una acción local pero no sistémica [22,32]. La razón de estas diferencias se desconoce aún, pero se ha planteado que la mayor actividad hemorrágica de las MPVSs de tipo PIII puede deberse a cuatro posibles mecanismos: a) la presencia de exositios en los dominios adicionales tipo disintegrina y rico en cisteína de las MPVSs tipo PIII que pueden dirigir a la toxina hacia blancos relevantes en la

microvasculatura, como integrinas del endotelio o proteínas de la membrana basal (MB); b) los dominios adicionales pueden contribuir a la alteración de mecanismos hemostáticos, como el efecto inhibidor de la agregación plaquetaria; c) las MPVSs tipo PIII son más resistentes a la inhibición por proteínas plasmáticas, como la α2-macroglobulina, que las tipo PI; d) la glicosilación de las MPVSs puede contribuir en la mayor actividad hemorrágica de las MPVSs de tipo PIII [13,33].

Existen estudios in vitro que han demostrado que los dominios adicionales tipo disintegrina y rico en cisteína (DC) de las MPVSs de tipo PII y PIII contienen exositios que determinan la unión de estas enzimas a dianas particulares de la matriz extracelular (MEC), como por ejemplo colágeno tipo I [34-38], colágeno tipo IV [36], colágeno tipo VI [37], integrina a2B1 [35,38], integrina avB3 [39], factor von Willebrand (vWF) [35,38], y proteínas que poseen el dominio A del vWF como colágenos asociados a los fibrilares con triple hélice interrumpida (FACITS) tipo XII y XIV y matrilinas 1, 3 y 4 [40]. También se ha demostrado in vitro la unión de MPVSs de tipo PIII con fibrinógeno, fibronectina y laminina [37]. Sin embargo, aún se desconoce la importancia de la unión in vivo a estas proteínas, y si dicha unión juega un papel en el mecanismo hemorrágico de estas toxinas. Recientemente, estudios de inmunohistoquímica han revelado un patrón distinto de distribución entre MPVSs hemorrágicas de tipo PI y PIII, mostrando una ubicación de esta última alrededor de la MB de diferentes estructuras en el tejido de la piel [41,42]. A pesar de la evidencia experimental de la unión de las PIII a componentes de la MEC in vitro y estructuras en tejidos in vivo, aún es necesario realizar estudios, principalmente en modelos in vivo, que permitan un análisis detallado y cuantitativo de la ubicación en los tejidos de las MPVSs hemorrágicas y no hemorrágicas de tipo PI, PII y PIII, con el fin de identificar el componente (s) clave (s) al cual se están uniendo las MPVSs y si estas diferencias están asociadas a la variación en la actividad hemorrágica.

1.3. Efectos biológicos de las MPVSs

Además de la acción hemorrágica de las MPVSs, algunas de estas proteinasas alteran la hemostasia por diferentes mecanismos. Existen MPVSs, especialmente no hemorrágicas de tipo PIII, que son activadoras de protrombina y del factor de coagulación X, lo que provoca el consumo del fibrinógeno y lleva al paciente a un estado de desfibrin(ogen)ación. Otras MPVSs afectan la hemostasia al interferir con la función plaquetaria, especialmente al inhibir la agregación plaquetaria, aunque también existen MPVSs con efecto agonista de la agregación. También se han descrito MPVSs con actividad fibrino(geno)lítica [43].

Estas toxinas también están implicadas en otras alteraciones que ocurren durante el envenenamiento ofídico como formación de ampollas, dermonecrosis y mionecrosis [44]. La formación de ampollas es debido a la degradación de proteínas en la unión epidemisdermis llevando a la separación de la epidermis [44]. Por otro lado, las MPVSs son capaces de producir mionecrosis probablemente debido a la isquemia que se desarrolla como consecuencia de la hemorragia y la interrupción del flujo sanguíneo [45], lo cual se ha visto que contribuye a una pobre regeneración muscular debido al daño y las alteraciones que ocurren en la microvasculatura [45,46].

Las MPVSs también participan en la respuesta inflamatoria, formación de edema y aumento de la permeabilidad vascular que ocurren durante el envenenamiento ofídico [22,44,47]. Además, contribuyen en la generación de dolor, que es característico de estos envenenamientos [48]. Estos procesos son debidos a un efecto directo de hidrólisis de componentes de la MEC y a la liberación de mediadores inflamatorios, como citoquinas e interleucinas, activación del complemento y reclutamiento de leucocitos [49–53]. Las MPVSs también pueden activar las MMPS endógenas mediante la escisión de su propéptido y la liberación de la forma activa, lo cual puede afectar los procesos de degradación y remodelamiento de la MEC [49,54].

Por otro lado, la hidrólisis de componentes de la MEC también puede llevar a la liberación de péptidos con diferentes actividades biológicas, como por ejemplo péptidos con actividad anti-angiogénica [55], e intereferir en la unión célula endotelial-MEC alterando la señalización en las adhesiones focales [53,56]. Estudios *in vitro* han demostrado que estas toxinas son capaces de inducir apoptosis secundaria al desprendimiento de las células, proceso conocido como anoikis [57,58]. Recientemente se ha descrito una metaloproteinasa

de tipo PIII capaz de alterar las uniones célula-célula por reubicación de la VE cadherina luego de la proteólisis de la proteína relacionada al receptor de lipoproteína de baja densidad tipo 5 y 6 (LRP5/6), lo cual podría contribuir con la actividad hemorrágica de estas toxinas [59].

Dada la gran variedad de efectos biológicos de las MPVSs y su participación en diferentes procesos en la patología del envenemaniento, es importante estudiar el efecto de estas toxinas sobre diferentes componentes de la microvasculatura, no solo para comprender el mecanismo de acción hemorrágico, sino también para estudiar otras acciones que puedan tener implicaciones sobre diferentes alteraciones que ocurren en el envenenamiento ofídico. Además, las MPVSs pueden convertirse en una herramienta importante para el estudio de otras enzimas con estructura y función similares, como lo son las MMPs, ADAMs y ADAMTs, las cuales juegan un papel importante en diferentes procesos fisiológicos y patológicos en mamíferos.

1.4. Estructura de la microvasculatura

Se ha descrito que la hemorragia inducida por las MPVSs se origina rápidamente y de manera explosiva en la microvasculatura, específicamente en capilares y vénulas pequeñas [46]. La microvasculatura se refiere a los vasos sanguíneos pequeños, con un diámetro igual o menor a 100 µm. Los tipos de vasos que conforman la microvasculatura son: arteriolas, capilares y vénulas post-capilares (VPC). Estos vasos sanguíneos están conformados por tres estructuras principales: una capa interna de células endoteliales, una MB circundante y una cantidad variada de células de músculo liso y pericitos de acuerdo con el tipo de vaso [60].

Las arteriolas poseen una mayor capa de células de músculo liso con uniones estrechas entre las células adyacentes, característica que les permite a estos vasos participar en el control de la resistencia vascular [61]. Los capilares son vasos con diámetro menor a 12 µm y su pared está conformada solamente por una capa de células endoteliales y una MB circundante, estructura que es importante para mantener la estabilidad de estos vasos. Las VPC poseen una menor cantidad de células de músculo liso de distribución irregular y

células denominadas pericitos, los cuales son un tipo de célula de músculo liso especializado. En las VPC es donde se dan procesos como respuesta inflamatoria, regulación de la permeabilidad vascular y extravasación de leucocitos [60].

Las células endoteliales forman una capa de epitelio plano simple que recubre internamente los vasos sanguíneos y cumplen funciones importantes en el mantenimiento de la hemostasia, control del tono vasomotor, participan en la respuesta inflamatoria e inmune, el control de la permeabilidad vascular y el intercambio de fluidos y macromoléculas en los tejidos [60]. El endotelio vascular, junto con la MB, conforman la principal barrera de permeabilidad vascular. La unión entre las células endoteliales está mediada por tres tipos de uniones: las uniones estrechas ("tight junctions"), conformadas principalmente por claudinas y ocludinas; las uniones adherentes ("adherens junctions"), conformadas por proteínas de adhesión transmembrana de la familia de las cadherinas y las uniones en hendidura ("gap junctions") las cuales permiten la comunicación bidireccional entre las células [62,63]. La VE cadherina es una proteína transmembrana que se expresa exclusivamente en las células endoteliales y tiene funciones importantes en la integridad microvascular [64,65] y en la regulación de la permeabilidad vascular [66,67]. Por otro lado, las adhesiones focales ("focal adhesions") median la unión de las células endoteliales con la MEC y están conformadas por proteínas de adhesión transmembrana de la familia de las integrinas [68].

Los pericitos son un tipo de célula de músculo liso especializado que forman una red de células embebidas dentro de la MB de las VPC, formando hendiduras u orificios entre las células adyacentes; aunque también se pueden encontrar en arteriolas, capilares y vasos sanguíneos de mayor tamaño [69,70]. Estas células están en estrecho contacto y comunicación con las células endoteliales y tienen funciones importantes en el mantenimiento de la integridad vascular, contracción vascular, regulación del flujo sanguíneo, remodelamiento de la MB durante el proceso inflamatorio, angiogénesis, desarrollo vascular y cicatrización de heridas [71–77].

La MB es una capa de MEC especializada, de 50-100 nm de espesor, que da soporte a las células endoteliales, y a otras células como las epiteliales, musculares, adiposas y de Schwann. Además de dar soporte estructural a estas células, la MB cumple funciones importantes en la organización de los tejidos, adhesión celular, comunicación celular, permeabilidad vascular; además, constituye un reservorio de factores de crecimiento y participa en procesos de mecanotransducción. La MB está constituida principalmente por redes independientes de colágeno tipo IV y laminina, las cuales se interconectan por medio del nidogén y proteoglicanos de heparán sulfato como el perlecan [78-82]. El colágeno tipo IV es el principal componente estructural de la MB, representando el 50% de las proteínas de esta estructura; además, es la única proteína de la MB que forma una red unida covalentemente y por lo tanto tiene un rol importante en la estabilidad mecánica de la MB [83–88]. Existen otras proteínas que se encuentran en menor concentración como agrina. fibulinas, colágenos VII, VIII, XV, XVIII, factor von Willebrand, entre otros [80-82,89]. Por otro lado, existen múltiples isoformas de los principales componentes de la MB, así como de los componentes en menor cantidad, por lo que la composición química de la MB varía entre los tejidos [89].

Existen muchas técnicas y modelos para el estudio de la microvasculatura y sus componentes. Uno de los modelos más utilizados es el estudio de la microvasculatura en el músculo cremáster de ratón. El músculo cremáster es un tejido de músculo estriado que recubre los testículos y tiene la particular característica de ser delgado y transparente, lo que permite obtener imágenes longitudinales de alta resolución de los vasos sanguíneos sin la necesidad de realizar cortes en el tejido. El uso de este modelo junto con la microscopía confocal ha permitido obtener imágenes de alta resolución en tres dimensiones con lo cual es posible realizar un análisis más detallado y cuantitativo de cada uno de los componentes de los vasos sanguíneos [71,90–93]. En el campo de la toxinología, el músculo cremáster ya ha sido utilizado para el estudio del efecto de las MPVSs hemorrágicas, otras toxinas y venenos en general por medio de microscopía intravital [46,94,95]. Sin embargo, estos estudios son descriptivos y no se ha profundizado en los efectos sobre los diferentes componentes de la microvasculatura, además no se ha utilizado la microscopía confocal.

1.5. Mecanismo de acción hemorrágico de las MPVSs

Aunque el mecanismo de acción hemorrágico de las MPVSs no está completamente elucidado, se ha demostrado que estas toxinas actúan principalmente sobre la microvasculatura por medio de dos mecanismos: hemorragia per diapédesis y hemorragia per rhexis, siendo la hemorragia per rhexis el mecanismo predominante [13,33,44]. La hemorragia per diapédesis ocurre por extravasación de eritrocitos a través de las uniones entre las células endoteliales, principalmente a nivel de vénulas, debido al proceso inflamatorio que se genera [96,97]. Recientemente se ha descrito un mecanismo por el cual las MPVSs podrían estar promoviendo la reubicación de la VE cadherina luego de la proteólisis del receptor LRP5/6, lo cual puede llevar a un aumento de la permeabilidad vascular y favorecer la salida de eritrocitos al intersticio; sin embargo, aún no se sabe si este mecanismo es suficiente para inducir hemorragia por sí solo o si es un mecanismo exclusivo de las MPVSs de tipo PIII, para la cual fue descrito [59]. La hemorragia per rhexis ocurre por ruptura de los vasos sanguíneos, principalmente capilares, lo que provoca una salida explosiva de eritrocitos [98–101]. Se ha planteado la posibilidad de que la diferencia entre estos dos tipos de mecanismos de hemorragia se relacione con el tipo de microvaso que se analice. Así, los estudios que han mostrado hemorragia per diapédesis han estudiado fundamentalmente vénulas, en tanto en los estudios en los que se ha descrito hemorragia per rhexis se ha analizado principalmente capilares [44]. Esto plantea la relevancia de analizar la acción de las MPVSs hemorrágicas comparativamente en vénulas y en capilares.

Estudios de microscopía intravital han demostrado que la hemorragia aparece en los primeros minutos después de la aplicación de una MPVS hemorrágica y se da principalmente en capilares y vénulas pequeñas de manera explosiva [31,46,95]. Estudios de microscopía electrónica en modelos *in vivo* han demostrado alteraciones en las células endoteliales y en la MB de la microvasculatura luego de la administración de MPVSs hemorrágicas [99,101], lo que haría pensar que las células endoteliales pueden ser el principal blanco de estas toxinas; sin embargo, estudios en cultivo celular han demostrado que el daño que pueden causar las MPVSs sobre células endoteliales no es tan rápido como el que ocurre *in vivo*, y más bien se observa un desprendimiento de las células endoteliales seguido de muerte celular

por apoptosis luego de varias horas [57,58,95]. Por otro lado, existen estudios que han demostrado la capacidad de estas toxinas de degradar componentes de la MB en diferentes modelos *in vitro* e *in vivo* [31,37,41,42,46,54,102–111]. Esto sugiere que el principal sitio de acción de las MPVSs hemorrágicas sería la MB, lo cual llevaría a un daño indirecto de las células endoteliales [44].

En este sentido, Gutiérrez *et al.* [13] postularon un mecanismo del efecto hemorrágico de estas toxinas sobre la microvasculatura en dos pasos: 1) inicialmente estas toxinas degradan proteínas de adhesión y componentes de la MB que rodea las células endoteliales de los capilares, lo cual afecta la interacción entre las células endoteliales y la MB y lleva a un debilitamiento de la estructura del capilar; 2) por otro lado, las fuerzas hemodinámicas normales, como la presión hidrostática y la fuerza de cizalla (*shear stress*), causan distensión y adelgazamiento de las células endoteliales de los capilares afectados, hasta que finalmente se pierde la integridad del capilar y se produce la extravasación de eritrocitos y otros componentes sanguíneos [13,33,112]. Esto explicaría el rápido daño que ocurre en las células endoteliales *in vivo*, en comparación al daño tardío que se ha observado en cultivos de células endoteliales.

1.5.1. Componentes de la MB como posibles blancos de las MPVSs hemorrágicas

Existen estudios que han demostrado la degradación por parte de MPVSs hemorrágicas sobre los cuatro componentes principales de la MB: colágeno tipo IV, laminina, nidogén y perlecan [31,37,41,42,46,54,103–111]. La mayoría de estos estudios se han realizado en modelos *in vitro* sobre el sustrato aislado [31,37,42,46,103–107], aunque más recientemente existe evidencia de la degradación de estos componentes en modelos *in vivo* [41,108,109,111]. Algunos de estos estudios han comparado las alteraciones inducidas por MPVSs hemorrágicas de tipo PI y PIII [41,42,107], así como MPVSs hemorrágicas y no hemorrágicas de tipo PI [109,111], con el fin de identificar diferencias que permitan explicar las variaciones en la actividad hemorrágica entre los tipos de MPVSs.

Al comparar la capacidad de degradación de componentes de la MB de una MPVS hemorrágica tipo PI, la BaP1 de *B. asper*, y una MPVS hemorrágica tipo PIII, la jararhagina de *Bothrops jararaca*, se observó en estudios inmunohistoquímicos que ambas toxinas disminuyen la tinción para laminina, nidogén y colágeno IV en capilares de músculo esquelético debido probablemente a la ruptura de los capilares por las fuerzas hemodinámicas y no necesariamente por una degradación directa de las MPVSs; mientras que en estudios *in vitro* se demostró una diferencia parcial en el patrón de degradación para laminina y nidogén, donde la jararhagina tiene una preferencia sobre el nidogén y genera sitios de corte diferentes a los generados por la BaP1, lo cual podría contribuir a la diferencia en la actividad hemorrágica de estas toxinas [107]. Sin embargo, este estudio se basó en efectos sobre matrigel[®], una preparación de MB solubilizada extraída del sarcoma de ratón Engelbreth-Holm-Swarm (EHS), y además no se estudió el efecto de estas dos toxinas sobre el colágeno tipo IV, ni otras proteínas de la MEC circundante.

En otro estudio que comparó las alteraciones inducidas por MPVSs hemorrágicas de tipo PI, la BnP1 de *Bothrops neuwiedi*, y tipo PIII, la jararhagina de *B. jararaca*, se demostró una disminución de la inmunotinción de colágeno IV y laminina en la microvasculatura de piel luego de la administración de ambas MPVSs, donde el mayor efecto observado fue el de la PIII sobre el colágeno IV [41]. Además, en este mismo estudio se demostró una colocalización de la jararhagina y la jararhagina C (dominio tipo disintegrina y rico en cisteína de la jararhagina) con el colágeno IV [41], lo cual da soporte a la hipótesis de que los dominios tipo disintegrina y rico en cisteína de las MPVSs tipo PIII pueden dirigir a la toxina hacia blancos relevantes en la microvasculatura lo cual contribuiría a la mayor actividad hemorrágica [13,33]. Sin embargo, este estudio no incluyó una MPVS de tipo PII, ni realizó un análisis cuantitativo y detallado de la degradación de los componentes de la MB y la colocalización de las toxinas con estos componentes.

Por otro lado, al comparar la capacidad de degradación de componentes de la MB de una MPVS hemorrágica tipo PI, la BaP1 de *B. asper*, y una MPVS no hemorrágica tipo PI, la leucurolysina de *Bothrops leucurus*, se demostró que existe una especificidad y un patrón de degradación diferente en modelos *in vivo* e *in vitro* para laminina, nidogén colágeno IV y perlecan, donde la BaP1 muestra una mayor capacidad para degradar colágeno IV, perlecan y en menor grado nidogén, en tanto la leucurolisina no degradó el colágeno IV [109]. En otro estudio, al comparar la capacidad de degradación de componentes de la MB de dos MPVSs tipo PI con diferente actividad hemorrágica, la BaP1 de B. apser y la BpirMP de Bothrops pirajai, se demostró que existen diferentes patrones de degradación para laminina y nidogén en modelos in vivo e in vitro, mientras que el colágeno IV fue degradado principalmente por la BaP1, lo que podría explicar la mayor capacidad hemorrágica de la BaP1 sobre la PpirMP [111]. Estos hallazgos hacen suponer que las diferencias en la actividad hemorrágica entre estas toxinas se pueden deber a las variaciones en la especificidad y los fragmentos de degradación de componentes de la MB, y principalmente a la diferente capacidad de degradar colágeno IV y perlecan. Estos estudios han incorporado herramientas como la proteómica de exudado inflamatorio [109] y la inmunodetección por western blot en homogenizados de tejidos [109,111], lo cual ha permitido estudiar efectos en modelos in vivo y ha brindado información muy útil en cuanto al mecanismo de acción de las MPVSs de tipo PI hemorrágicas y no hemorrágicas; sin embargo, aún no existe un estudio comparativo entre diferentes MPVSs hemorrágicas de tipo PI, PII y PIII que utilice estas herramientas.

Recientemente los análisis de proteómica han constituido una herramienta valiosa para el estudio del mecanismo de acción hemorrágico de las MPVSs, permitiendo un estudio más amplio del fenómeno y la identificación de nuevos sustratos [108,109,113,114]. Estudios de proteómica del exudado inflamatorio inducido por BaP1 en músculo esquelético han evidenciado la degradación de colágenos no fibrilares como los tipos VI, XII, XIV, XV y XVI [108,109]. Adicionalmente, existen estudios *in vitro* han demostrado la capacidad de las MPVSs de tipo PIII de unirse y degradar al colágeno tipo VI [37,115]. El colágeno VI tiene una función importante en la estabilidad y organización estructural de los vasos al conectar componentes de la MB con colágenos fibrilares de la MEC [116]. Por lo tanto, la degradación del colágeno tipo VI y otras proteínas de la MEC circundante también pueden ser clave o participar en el mecanismo de acción hemorrágico de estas toxinas.

A pesar de la evidencia experimental que demuestran la capacidad de estas toxinas de actuar sobre diferentes componentes de la MB y la MEC [31,37,41,42,46,54,102–111] y algunas diferencias entre MPVSs hemorrágicas y no hemorrágicas de tipo PI [109,111], y MPVSs hemorrágicas de tipo PI y PIII [41,42,107], aún no se conoce en detalle los componentes de la MB sobre los que actúan las MPVSs que sean responsables de la hemorragia y si existen diferencias entre los tipos de MPVSs que permitan explicar las variaciones en la actividad hemorrágica [33]. Por otro lado, la mayoría de los estudios se basan en la degradación de proteínas de la MB y MEC *in vitro*, por lo que existe la necesidad de contar con más herramientas, específicamente modelos *in vivo*, para el estudio del mecanismo de acción de las MPVSs que permitan un análisis más detallado de los efectos de estas toxinas sobre la MB y otros componentes de la microvasculatura.

El estudio comparativo de las alteraciones inducidas por MPVSs sobre componentes de la microvasculatura utilizando el músculo cremáster y la microscopía confocal, constituye una estrategia novedosa en el campo de las MPVSs para estudiar la distribución y colocalización de las toxinas, así como la degradación de los componentes de la MB y otros efectos sobre la microvasculatura. Por otro lado, es necesario realizar estudios comparativos entre diferentes MPVSs hemorrágicas de tipo PI, PII y PIII en modelos *in vivo* que incorporen herramientas como la proteómica de exudado inflamatorio y la inmunodetección por western blot en homogenizados de tejidos, lo cual puede brindar información muy útil en cuanto al mecanismo de acción de estas MPVSs.

Ante este panorama, el presente trabajo de investigación compara las alteraciones inducidas por MPVSs hemorrágicas de tipo PI, PII y PIII sobre componentes de la MB y MEC circundante, principalmente sobre colágeno tipo IV, colágeno VI, laminina y nidogén, así como alteraciones sobre otros componentes de la microvasculatura por medio de modelos *in vivo* que utilizan piel, músculo cremáster y exudado inflamatorio de gastronemio y las técnicas de proteómica, inmunoquímica e inmunofluorescencia, con el fin de identificar diferencias entre los tipos de MPVSs que permitan explicar las variaciones en la actividad hemorrágica y contribuir así a la comprensión del mecanismo de acción de estas toxinas. Además, en el presente trabajo se estudia el efecto de las fuerzas hemodinámicas sobre las

alteraciones de la MB y otros componentes de la microvasculatura utilizando un modelo *ex vivo* que elimina el flujo sanguíneo y se compara dichas alteraciones de acuerdo con el tipo de vaso sanguíneo (*i.e.* capilares, VPC y arteriolas). Finalmente, se estudia el patrón de distribución y la capacidad de unión de diferentes tipos de MPVSs hemorrágicas y no hemorrágicas a componentes de la MB vascular.

2. OBJETIVOS

Objetivo General

Estudiar alteraciones inducidas por metaloproteinasas de venenos de serpientes de tipo PI, PII y PIII con distinta actividad hemorrágica sobre proteínas de la membrana basal y otros componentes de la microvasculatura por medio de modelos *ex vivo* e *in vivo*, con el fin de identificar diferencias entre los tipos de MPVSs que permitan explicar las variaciones en la actividad hemorrágica y los posibles mecanismos de inducción de hemorragia.

Objetivos Específicos

- Comparar el patrón de distribución y la capacidad de unión de diferentes tipos de MPVSs a componentes de la membrana basal vascular, por medio de pruebas de inmunofluorescencia y microscopía confocal en un modelo ex vivo que utiliza músculo cremáster murino.
- 2. Comparar las alteraciones inducidas por distintas MPVSs sobre los diferentes componentes de la microvasculatura (*i.e.* células endoteliales, membrana basal, células de músculo liso/pericitos) en los diferentes tipos de vasos (*i.e.* arteriolas, capilares, VPC), por medio de pruebas de inmunofluorescencia y microscopía confocal en modelos *ex vivo* e *in vivo* que utilizan músculo cremáster murino.
- 3. Identificar las principales proteínas de la membrana basal y matriz extracelular circundante que son degradadas *in vivo* por distintas MPVSs y veneno completo, por medio de pruebas inmunoquímicas, de inmunofluoresecia y estudios de proteómica en diferentes modelos murinos que utilizan piel, músculo cremáster y exudado inflamatorio de gastronemio.

3. MATERIALES Y MÉTODOS

3.1. Obtención de las metaloproteinasas

Se trabajó con tres MPVSs hemorrágicas de venenos de serpientes: a) BaP1, una MPVS tipo PI del veneno de *Bothrops asper* [22,117], b) BlatH1, una MPVS tipo PII dimérica del veneno de *Bothriechis lateralis* [118] y c) CsH1, una MPVS tipo PIII del veneno de *Crotalus simus* [119]. Estas tres MPVSs tienen diferente actividad hemorrágica, siendo el orden de potencia hemorrágica el siguiente: BlatH1 > CsH1 > BaP1. Para los estudios de distribución de las toxinas se utilizó también la Basparina A [27], una MPVS tipo PIII no hemorrágica del veneno de *Bothrops asper*. Los venenos fueron proporcionados por el serpentario del Instituto Clodomiro Picado y constituyen un liofilizado de un pool de veneno obtenido de al menos 20 especímenes adultos colectados en diversas regiones del país.

La BaP1 se aisló mediante una cromatografía de intercambio iónico en una columna de carboximetil (CM)-Sepharose[®], seguido de cromatografía de afinidad en una columna de Affi Gel Blue[®] [22,117]. La BlatH1 se aisló mediante una combinación de cromatografía de intercambio iónico en una columna de dietilaminoetil (DEAE)-Sepharose[®], seguido de cromatografía de interacciones hidrofóbicas en una columna de Phenyl-Sepharose[®], y finalmente cromatografía de filtración en gel en una columna de Superdex[®] 200 10/300GL en un equipo de cromatografía líquida rápida de proteínas (FPLC, por sus siglas en inglés) [118]. La CsH1 se aisló mediante una combinación de cromatografía de intercambio iónico en una columna de DEAE-Sepharose[®], seguido de cromatografía de filtración en gel en una columna de JIT8]. La Basparina A fue proporcionada por la Dra. Alexandra Rucavado del Instituto Clodomiro Picado y se aisló siguiendo el protocolo descrito por Loría *et al.* [27].

3.2. Animales de experimentación

Para la mayoría de las pruebas con animales se utilizaron ratones CD-1, de ambos sexos, con un peso entre 18-20 g, provenientes del Bioterio del Instituto Clodomiro Picado, Universidad de Costa Rica. Para las pruebas que utilizan el músculo cremáster como modelo de estudio, se utilizaron ratones machos C57BL/6, con un peso entre 20-25 g, provenientes del Laboratorio Charles River (Cambridge, Reino Unido) y el Laboratorio de Ensayos Biológicos de la Universidad de Costa Rica. El uso de animales para la ejecución del presente trabajo fue aprobado por el Comité Institucional para el Cuido y Uso de Animales, de la Universidad de Costa Rica, bajo los números CICUA 19-09 y CICUA 003-15, y por el Consejo de Bienestar y Ética de los Animales (AWERB, por sus siglas en inglés), de la Universidad Queen Mary, Londres, Reino Unido. Los animales se mantuvieron bajo condiciones estándar de temperatura y humedad controlada, ciclos de luz y oscuridad de 12 horas, suministro de comida y agua *ad libitum*, de acuerdo con los Principios Internacionales para la Investigación Biomédica con Animales (CIOMS, por sus siglas en inglés) y la legislación de Reino Unido para la protección animal.

3.3. Distribución de las toxinas y efectos de las MPVSs sobre la microvasculatura de músculo cremáster de ratón

Para el estudio de la distribución e inmunolocalización de las MPVSs con componentes de la MB vascular, y los efectos de estas toxinas sobre los diferentes componentes de la microvasculatura, se decidió utilizar el músculo cremáster debido a que es un tejido delgado y transparente, lo cual permite trabajar con el tejido completo y obtener imágenes longitudinales de los vasos sanguíneos de alta resolución por medio de la microscopía confocal.

3.3.1. Exposición del músculo cremáster a las toxinas

3.3.1.1. Distribución de las toxinas en el tejido y estudios de colocalización

Para poder visualizar las MPVSs en el tejido, éstas fueron marcadas con el fluorocromo Alexa Fluor 647 utilizando el kit comercial Microscale Protein Labeling Kit (Molecular Probes A30009). Para determinar si el marcaje con el fluorocromo afecta la actividad enzimática de las MPVSs, se cuantificó la actividad proteolítica de las toxinas

marcadas y sin marcar sobre gelatina, a diferentes tiempos durante 24 h, utilizando el kit comercial EnzChek Gelatinase Assay Kit (Molecular Probes E-12055).

Grupos de tres ratones machos C57BL/6 (20-25 g) fueron sacrificados por dislocación cervical. Inmediatamente se removió el músculo cremáster y se incubó por 15 minutos, a temperatura ambiente, con las toxinas marcadas: 30 μ g de BaP1, 3.5 μ g de BlatH1 o 15 μ g de CsH1, disueltas en 100 μ l de solución amortiguadora de fosfatos (PBS). Adicionalmente, se utilizaron tejidos control los cuales fueron incubados con cada una de las toxinas sin marcar. Las toxinas fueron aplicadas en la superficie del tejido por medio de una micropipeta. Las dosis fueron seleccionadas de manera tal que inducen una hemorragia de intensidad similar en el músculo cremáster, como se describió previamente por microscopía intravital para la BaP1 [46]. En el caso de la Basparina A, se utilizó 6.5 μ g y se comparó con una cantidad equimolar de la CsH1 (5 μ g), tomando en consideración que es una cantidad de la CsH1 que puede ser detectada unida a la MB vascular. Luego del período de incubación, se realizaron lavados con PBS para eliminar las toxinas que no se unieron a estructuras del tejido. Posteriormente, los tejidos fueron fijados con 4% de paraformaldehído disuelto en PBS, por 30 min, a 4°C, y se continuó con el proceso de inmunotinción del colágeno tipo IV, el cual se detalla en la sección 3.3.2.

3.3.1.2. Modelo ex vivo (i.e. sin flujo sanguíneo)

Grupos de cuatro ratones machos C57BL/6 (20-25 g) fueron sacrificados por dislocación cervical. Inmediatamente se removió el músculo cremáster y se incubó a temperatura ambiente con diferentes cantidades de BaP1 o CsH1, disueltas en 100 µl de PBS. Las toxinas fueron aplicadas en la superficie del tejido por medio de una micropipeta. Adicionalmente, se utilizaron tejidos control los cuales fueron incubados con 100 µl de PBS. Inicialmente se estudiaron los efectos dosis y tiempo dependientes de la BaP1 sobre el colágeno tipo IV de la MB vascular, para lo cual se evaluaron tres dosis de la BaP1 (10, 30 y 100 µg) y dos tiempos de incubación (5 y 15 min). La dosis de 30 µg de la BaP1 y el tiempo de incubación de 15 min fueron seleccionados para el resto de los análisis. En el caso de la CsH1, se seleccionó una dosis de 15 µg, la cual induce una hemorragia de intensidad

similar en el músculo cremáster a la causada por 30 µg de BaP1, como se describió previamente por microscopía intravital [46]. Esta relación de masa 2:1 de las MPVSs corresponde a una relación molar aproximada de 5:1. Luego del período de incubación, se realizaron lavados de los tejidos con PBS y se fijaron con 4% de paraformaldehído disuelto en PBS, por 30 min, a 4°C. En el caso de la inmunotinción para laminina se utilizó metanol como solución fijadora. Posteriormente, se continuó con el proceso de inmunotinción para visualizar diferentes componentes de la microvasculatura, como se detalla en la sección 3.3.2.

3.3.1.3. Modelo in vivo (i.e. con flujo sanguíneo)

Grupos de cuatro ratones machos C57BL/6 (20-25 g) fueron anestesiados con ketamina (35 mg/kg) / xilazina (15 mg/kg). Posteriormente, se administró por vía intrasescrotal 60 µg de BaP1 o 30 µg de CsH1, disueltas en 300 µl de PBS. Esta dosis corresponde al doble de la dosis utilizada en el modelo *ex vivo*, ya que cada ratón posee dos músculos cremáster. Como control se administró 300 µl de PBS. Los animales fueron sacrificados por dislocación cervical 15 min después de la administración de las toxinas. Inmediatamente se removió el músculo cremáster, se lavó con PBS y se fijó con 4% de paraformaldehído disuelto en PBS, por 30 min, a 4°C. Posteriormente, se continuó con el proceso de inmunotinción para visualizar diferentes componentes de la microvasculatura. como se detalla en la sección 3.3.2.

3.3.2. Inmunotinción del músculo cremáster

Luego del proceso de fijación, se realizó un montaje para inmunotinción del tejido completo siguiendo el protocolo descrito por Wang *et al.* [91]. Brevemente, los tejidos fueron incubados en una solución bloquedora y permeabilizadora (12.5% de suero de cabra, 12.5% de suero fetal bovino y 0.5% de Tritón X-100, disuelto en PBS), por 4 h, a temperatura ambiente, con agitación constante. Posteriormente, el tejido fue incubado por 48 h a 4°C, con alguno de los siguientes anticuerpos primarios contra componentes de la MB: anticuerpo policlonal de conejo anti-colágeno tipo IV (Abcam ab19808), anticuerpo policlonal de conejo anti-laminina

(Abcam ab11575). Al mismo tiempo los tejidos fueron incubados con un anticuerpo monclonal de ratón anti-actina a del músculo liso (aSMA, por sus siglas en inglés) marcada con el fluorocromo Cy3 (Clone 1A4, Sigma C6198) y un anticuerpo monoclonal de rata anti-VE cadherina (clon BV14, eBioscience 14-1442) para visualizar las células del músculo liso/pericitos y las uniones endoteliales célula-célula, respectivamente. El anticuerpo anti-VE cadherina fue previamente marcado con el fluorcromo Alexa 647 utilizando el kit comercial Monoclonal Antibody Labeling kit (Molecular Probes A20186). Después del período de incubación con los anticuerpos primarios, los tejidos fueron lavados con PBS e incubados por 4 h a 4ºC con un anticuerpo policional de cabra anti-IgG (H+L) de conejo marcado con Alexa 488 (Invitrogen A11034) para poder visualizar los componentes de la MB. Todos los anticuerpos fueron diluidos en PBS con 10% de suero fetal bovino. La dilución de los anticuerpos se puede ver en la sección de metodología en la publicación correspondiente (Artículo II). Después de 4h de incubación con el anticuerpo secundario, los tejidos fueron lavados con PBS. El tejido completo fue montado en un portaobjetos, se colocó PBS y luego un cubreobjetos, para poder observarlo en un microscopio confocal como se detalla en la sección 3.3.3.

3.3.3. Análisis del tejido por microscopía confocal

Los tejidos inmunomarcados, como se describió en la sección anterior, fueron observados por medio de dos microcopios: 1) un microcopio confocal de escaneo laser Zeiss LSM 5 Pascal incorporando un lente objetivo 10X (apertura numérica 0.3), un lente objetivo 40X (medio de inmersión agua, apertura numérica 0.8) y un lente objetivo 63X (medio de inmersión aceite, apertura numérica 1.4), y 2) un microscopio confocal de escaneo laser Olympus Fluoview FV1000 incorporando un lente 40X (medio de inmersión aceite, apertura numérica 1.4). El primer microscopio fue el que se utilizó durante la pasantía, y con éste se obtuvieron los resultados de la distribución de las tres MPVSs hemorrágicas y los efectos de la BaP1 sobre la microvasculatura. El segundo microscopio fue el que se utilizó en Costa Rica para continuar con los estudios, y con éste se obtuvieron los resultados de la distribución de la CsH1 sobre la miscrovasculatura.

Se tomaron imágenes tridimensionales de arteriolas, capilares y VPC por medio de un escaneo secuencial de los diferentes canales, a una resolución de 1024 x 1024 dpi, lo cual corresponde a un tamaño de voxel de 0.22 x 0.22 x 1.18 μ m y 0.31 x 0.31 x 0.57 μ m en los planos X x Y x Z, para las imágenes obtenidas con los microscopios Zeiss y Olympus, respectivamente. En el caso de las imágenes que se tomaron con el lente objetivo 63X para los estudios de colocalización, el tamaño de voxel corresponde a 0.14 x 0.14 x 0.38 μ m en los planos X x Y x Z. Las imágenes de VPC y arteriolas (vasos con un diámetro entre 20-45 μ m) se tomaron desde el centro del vaso hasta el borde superior, a lo largo del eje longitudinal (200 μ m de longitud); es decir, imágenes de semi-vasos. Con respecto a los capilares (vasos con un diámetro menor a 10 μ m), se tomaron desde el borde inferior hasta el borde superior, a lo largo del eje longitudinal (200 μ m de longitud); es decir, imágenes de vasos completos. En cada experimento, las imágenes de los tejidos tratados y control se tomaron bajo las mismas condiciones y ajustes del software.

3.3.3.1. Análisis de colocalización

Se tomaron al menos cuatro imágenes de arteriolas, capilares y VPC por tejido. La reconstrucción de las imágenes tridimensionales y el análisis de colocalización de las MPVSs con el colágeno tipo IV de la MB vascular se realizó por medio del software IMARIS x64 7.4.2, el cual emplea el fundamento desarrollado por Costes *et al.* [120] para el análisis de colocalización. Se calculó el umbral para cada marcaje (toxina marcada y colágeno tipo IV) con la función automática del software, para ello se definió una región de interés de 10 en la intensidad de pixel. El programa IMARIS analiza la intensidad de cada marcaje por voxels. Un voxel corresponde a un prisma con el pixel en la base y el espesor de la sección confocal en la altura. Los resultados de colocalización se expresaron de dos formas: 1) el porcentaje de material colocalizado, que toma en cuenta el número de voxels colocalizados con respecto al total de voxels, y 2) el coeficiente de correlación de Pearson, que refleja la correlación entre las intensidades de los voxels colocalizados, es decir toma en cuenta que la intensidad de las dos etiquetas varía de manera proporcional. El coeficiente de Pearson varía entre +1 y -1, donde los valores cercanos a 1 indican una correlación directa, y los valores cercanos a 0 indican que no hay correlación. En este tipo de estudios es

importante analizar estos dos valores debido a que el porcentaje de colocalización puede sobreestimar el grado de colocalización, mientras que el coeficiente de Pearson puede subestimar el grado de colocalización [120].

3.3.3.2. Análisis de los efectos de las MPVSs sobre los diferentes componentes de la microvasculatura

Se tomaron al menos cinco imágenes de arteriolas y VPC, y ocho imágenes de capilares por tejido. Posteriormente, las imágenes se analizaron por medio del software Image J. Se calculó la intensidad de la fluorescencia total (valor promedio en la escala de grises de todos los pixeles en el área analizada) para la inmunotición de los componentes de la MB en arteriolas, capilares y VPC. Por otro lado, se calculó el tamaño y densidad de espacios entre las células de músculo liso y pericitos adyacentes en arteriolas y VPC. Las imágenes tridimensionales se reconstruyeron por medio del software IMARIS x64 7.4.2. En el caso del análisis de las uniones endoteliales, éste se hizo de manera cualitativa a partir de la imagen tridimensional reconstruida para la inmunotinción de VE caherina.

3.4. Ensayo de permeabilidad vascular in vivo

Para evaluar la permeabilidad vascular inducida por las MPVSs, se siguió el protocolo descrito por Radu y Chernoff [121], con algunas modificaciones. Brevemente, grupos de cinco ratones CD1 (18-20 g) recibieron 200 µl del colorante de Azul de Evans (6 mg/ml, disuelto en PBS) por vía intravenosa en la vena lateral de la cola. Después de 5 min, se administró 2 µg de BaP1 o 1 µg de CsH1, disueltos en 50 µl de PBS, por vía intradérmica en la región abdominal ventral. Estas dosis fueron seleccionadas de manera tal que no induzcan hemorragia en la piel, para evitar el desarrollo de una lesión hemorrágica que pudiera interferir con la medición de la extravasación del Azul de Evans, y con el fin de mantener la misma proporción de masa (2:1) utilizada en el modelo *in vivo* con músculo cremáster. Se contó con un grupo control, el cual recibió 50 µl de PBS por vía intradérmica. Después de 15 min de la administración de las MPVSs, los ratones fueron sacrificados por dislocación cervical, se retiró su piel, se tomó una foto del área azul, lo cual corresponde al plasma extravasado, y se determinó el área por medio del programa Image J^{**}.

3.5. Degradación de proteínas de la MB y MEC en modelos in vivo

3.5.1. Obtención de homogenizados de piel

Grupos de cinco ratones CD1 (18-20 g) recibieron 75 µg de BaP1, 1,5 µg de BlatH1 o 35 µg de CsH1, disueltos en 100 µl de PBS, por vía intradérmica en la región ventral abdominal. Las dosis fueron seleccionadas de manera tal que indujeron una hemorragia de intensidad y área similar en la piel inyectada. El grupo control recibió 100 µl de PBS. Después de 15 min de la administración de las MPVSs, los ratones fueron sacrificados por dislocación cervical, se retiró su piel, y se tomó un área de 12 mm de diámetro en el sitio de la inyección. Todos los fragmentos de piel para cada tratamiento se colocaron juntos en nitrógeno líquido y se pulverizaron hasta que se obtuvo partículas finas. Cada homogenizado se resuspendió en 1,5 ml de solución de extracción (25 mM Tris-HCl, 150 mM NaCl, 8 M urea, 40 mM EDTA, 1% Triton X-100, 0,1% SDS, una tableta de inhibidor de proteasa de Roche por cada 10 ml, pH 7,4) y se mantuvo en agitación por una hora a 4°C. Posteriormente, las muestras se centrifugaron a 5200 g durante 5 min, se tomó el sobrenadante y se diluyó 1:2 con agua desionizada. Estas muestras se almacenaron en alícuotas a -70°C hasta el momento en que se realizó el análisis inmunoquímico, como se detalla en la sección 3.5.3.

3.5.2. Obtención del exudado inflamatorio de gastronemio

Grupos de cinco ratones CD1 (18-20 g) recibieron 75 µg de BaP1, 3 µg de BlatH1 o 35 µg de CsH1, disueltos en 50 µl de PBS, por vía intramuscular en el gastrocnemio derecho. Las dosis fueron seleccionadas de manera tal que indujeron un grado similar de hemorragia en el músculo. El grupo control recibió 50 µl de PBS. Después de 15 min de la administración de las MPVSs, los ratones fueron sacrificados por dislocación cervical. Inmediatamente, se realizó una incisión de 5 mm en la piel que cubría el músculo inyectado y se introdujo un tubo capilar heparinizado para recoger el exudado de la lesión. Se obtuvo un volumen aproximado de 20-50 µl de exudado de cada ratón. Las muestras de exudado se mezclaron para cada tratamiento, se liofilizaron y se almacenaron a -70°C para el análisis inmunoquímico y proteómico, como se detalla en las secciones 3.5.3 y 3.5.4. En el caso del estudio con el veneno completo de *B. asper*, se utilizó el mismo modelo descrito anteriormente utilizando una dosis de 50 µg del veneno, y se tomaron muestras de exudado a las 1, 6 y 24 horas después de la administración del veneno.

3.5.3. Inmunodetección de proteínas de la MB y MEC en el homogenizado de piel y exudado inflamatorio

Se tomó un volumen de 20 µl de cada muestra del sobrenadante del homogenizado de piel, o 20 µl con 100 µg de proteína de cada muestra de exudado, y se separó en condiciones reductoras por electroforesis en gel de poliacrilamida con dodecilsulfato sódico (SDS-PAGE) en gradiente del 4 al 15%, seguido de una transferencia a membranas de nitrocelulosa. La inmunodetección se realizó incubando las membranas durante la noche, a 4°C, bajo agitación, con cada uno de los siguientes anticuerpos primarios: anticuerpo policional de conejo anti-colágeno tipo VI (Millipore AB7821), anticuerpo policional de conejo anti-colágeno tipo IV (Abcam ab6586 y ab19808), anticuerpo policional de conejo anti-laminina (Abcam ab11575 y Thermo PA1-32130), anticuerpo policional de conejo antinidógen 1 (Abcam ab14511), anticuerpo policional de conejo anti-colágeno tipo I (Abcam ab21286), anticuerpo policional de conejo anti-fibronectina (Abcam ab2413). La dilución de los anticuerpos se puede ver en la sección de metodología en las publicaciones correspondientes (Artículos I y III). Se utilizó el anticuerpo anti-gliceraldehído-3-fosfato deshidrogenasa (GAPDH) (Abcam ab9485) como control de carga para las muestras provenientes del homogenizado de piel. Seguidamente, se realizó una incubación con anticuerpos anti-inmunoglobulinas conjugados con peroxidasa (Jackson ImmunoResearch), según el tipo de anticuerpo primario. La reacción se detectó utilizando el sustrato quimioluminiscente Lumi-Ligth (Roche). Las imágenes fueron obtenidas con el sistema ChemiDoc XRS + (BioRad) y el análisis se realizó con el software ImageLab (BioRad).

3.5.4. Análisis de proteómica del exudado inflamatorio

Los exudados liofilizados se enviaron al laboratorio del Dr. Jay Fox de la Universidad de Virginia, U.S.A., para el análisis por espectrometría de masas (MS, por sus siglas en inglés). Brevemente, las muestras se resuspendieron en agua y 20 µg de proteína fueron
separados por electroforesis SDS-PAGE en gradiente del 4-20%. Los geles se tiñeron con Azul Brillante de Coomassie y cada carril se cortó en diez secciones de igual tamaño. Cada fragmento de gel se trató con tripsina (20 ng/µl), previa reducción (DTT 10 mM) y alquilación de proteínas (50 mM de yodoacetamida). Los péptidos trípticos se extrajeron del gel para el análisis de MS por medio de una solución de acetonitrilo al 50% y ácido fórmico al 5%. Se realizó un análisis de cromatografía líquida masas/masas (LC/MS/MS, por sus siglas en inglés) en un equipo Thermo Electron Orbitrap Velos ETD. El detalle del ajuste de las variables del sistema de espectrometía de masas se puede ver en la sección de metodología en las publicaciones correspondientes (Artículos I y III). Los datos obtenidos se analizaron por medio del programa Sequest en Proteome Discoverer 1.4.1 contra la base de datos Uniprot Mouse. Los resultados se exportaron a Scaffold (versión 4.3.2, Proteome Software Inc., Portland, OR) para validar identificaciones de péptidos y proteínas basadas en MS/MS, y para visualizar múltiples conjuntos de datos de una manera comprensiva. La cuantificación relativa de las proteínas se realizó sumando todos los datos de los 10 fragmentos de gel para cada muestra en Scaffold y luego mostrando el valor cuantitativo del programa. Este valor da cierta medida de abundancia relativa entre las proteínas generadas a partir del análisis por MS para una muestra de exudado particular y permite una comparación cuantitativa relativa entre una proteína específica presente en diferentes muestras. Adicionalmente, se analizaron porciones de los datos de manera manual para determinar si los espectros de masas se derivaban de proteínas que emigraban en el gel a una masa inferior a la esperada, con el fin de determinar porcentajes de degradación de las proteínas.

3.6. Análisis estadístico

Cuando fue posible, los resultados fueron expresados como el promedio \pm el error estándar de la muestra (EEM). Para comparar medias entre grupos experimentales se realizó una prueba de Análisis de Variancia (ANOVA) de una vía y ANOVA multifactorial, cuando se comparó más de un factor, con una prueba de Tukey como estudio *post-hoc*. Los supuestos de normalidad y homogeneidad de varianza se evaluaron por medio de las pruebas de

Shapiro Wilk y Levene, respectivamente. Se utilizó el programa IBM SPSS® Stadistics 22.0 y GraphPad Prims 6. Se consideró significativo un valor de p < 0.05.

4. RESULTADOS Y DISCUSIÓN

4.1. Distribución de diferentes tipos de MPVSs en la microvasculatura

4.1.1. Comparación de MPVSs hemorrágicas de tipo PI, PII y PIII

Cuando se comparó la distribución de cantidades equi-hemorrágicas de tres diferentes tipos de MPVSs: BaP1 (PI), BlatH1 (PII) y CsH1 (PIII), sobre el músculo cremáster de ratón aislado, se observaron diferencias en la distribución de las toxinas en el tejido (Figura 2A y 2B, artículo I). Las MPVSs de tipo PII y PIII se localizaron en la membrana basal de los vasos sanguíneos; mientras que la MPVS de tipo PI se distribuyó de manera más difusa en el tejido, con predominio alrededor de los vasos sanguíneos. El análisis de colocalización de las MPVSs con el colágeno tipo IV de la MB vascular (Figura 2C y 2D, artículo I) revela porcentajes de colocalización de alrededor del 40% para las MPVSs de tipo PII y PIII, tanto en capilares, como en arteriolas y VPC (coeficiente de Pearson alrededor de 0.4); mientras que para la MPVS tipo PI el porcentaje fue menor al 7% (coeficiente de Pearson menor a 0.07). No se encontraron diferencias significativas entre los porcentajes de colocalización para las MPVSs de tipo PII y PIII, ni entre el tipo de vaso sanguíneo, lo cual indica que la distribución de estas dos toxinas es muy similar en la microvasculatura.

Los tejidos incubados con las toxinas sin marcar no mostraron señal fluorescente, con lo cual se descarta que la señal observada para la BaP1 sea inespecífica. Además, la actividad proteolítica sobre gelatina de las MPVSs marcadas con Alexa 647 se mantuvo entre un 75-85% en comparación con la actividad de las MPVSs sin marcar (Figura 1), lo cual demuestra que luego del marcaje las enzimas conservaron la actividad proteolítica. Por otro lado, en estudios de interferencia de unión, donde la toxina marcada se agregó en los tejidos pre-incubados con la toxina sin marcar, se observó una disminución en la intensidad fluorescente para la toxina marcada, lo cual demuestra que la unión es específica hacia algún blanco en la MB.



Figura 1. Actividad proteolítica sobre gelatina de las MPVSs BaP1, BlatH1 y CsH1 marcadas con el fluorocromo Alexa 647. A) Se cuantificó la actividad proteolítica sobre gelatina de las MPVSs BaP1 (1 μg), BlatH1 (5 μg) y CsH1 (5 μg) sin marcar y marcadas con Alexa 647 a diferentes tiempos durante 24 h, por medio del kit comercial EnzChek Gelatinase Assay Kit (Molecular Probes E-12055). Los resultados muestran el promedio de tres repeticiones. U.I.F.: Unidades de Intensidad Fluorescente. B) Los resultados muestran el porcentaje de actividad proteolítica sobre gelatina para cada enzima marcada con el fluorocromo Alexa 647 con respecto a la enzima sin marcar.

Un estudio previo ya había demostrado un patrón de distribución distinto entre MPVSs hemorrágicas de tipo PI y PIII, mostrando una localización de esta última alrededor de diferentes estructuras de la piel, incluyendo capilares y vénulas en la hipodermis y fibras musculares [41]. Recientemente, resultados similares fueron obtenidos en otro estudio que comparó la localización de estas mismas MPVSs en un modelo *in vitro* que simula estructuras de capilares en 3D [56], y otro estudio que comparó la distribución dos MPVS hemorrágicas de *B. atrox* de tipo PI y PIII en piel [42]. Los resultados obtenidos en el modelo *ex vivo* de músculo cremáster coinciden con estos estudios, y muestran las diferencias en la localización de las tres MPVSs en la MB vascular de una manera detallada, y con un enfoque morfométrico cuantitativo, para cada tipo de microvaso (i.e. capilares, arteriolas y VPC).

Además, se muestra por primera vez que una MPVS hemorrágica de tipo PII se distribuye de manera similar que las MPVS hemorrágicas de tipo PIII.

Estudios in vitro han demostrado que el dominio adicional rico en cisteína de las MPVSs de tipo PIII contienen exositios que median la unión con proteínas de la MEC, como por cjcmplo con colágeno tipo I [35,38], integrina $\alpha 2\beta 1$ [35,38], integrina $\alpha \nu \beta 3$ [39], vWF [35,38], y proteínas que poseen el dominio A del vWF como colágenos FACITS tipo XII y XIV y matrilinas 1, 3 y 4 [40]. También se ha demostrado que los dominios tipo disintegrina pueden interaccionar in vitro con colágenos tipo I [34,36,122] y tipo IV [36]. Además, Baldo et al. [41] demostraron en un modelo in vivo que el fragmento DC de la jararhagina, una MPVS tipo PIII, era capaz de unirse a estructuras alrededor de los vasos sanguíneos [41]. Por lo tanto, se ha postulado que la mayor actividad hemorrágica de las MPVSs de tipo PIII puede deberse en parte a la presencia de exositios en los dominios adicionales, los cuales puede dirigir a la toxina hacia blancos relevantes en la microvasculatura [13,33]. Los resultados de colocalización obtenidos en el modelo ex vivo de músculo cremáster apoyan esta hipótesis, e indican que tanto el fragmento tipo disintegrina como el rico en cisteína pueden ser claves en la interacción con componentes de la MB vascular, lo que puede contribuir a la mayor actividad hemorrágica de las MPVSs de tipo PII y PIII, en comparación a las de tipo PI. Sin embargo, la diferente actividad hemorrágica entre la BlatH1 (PII) y la CsH1 (PIII) sugiere que la unión a la MB vascular no sería el único determinate en la potencia hemorrágica. En este sentido, la capacidad proteolítica, las diferencias en los exositios y la estabilidad de las toxinas en el tejido pueden jugar también un papel importante en la potencia hemorrágica de las MPVSs.

Adicionalmente, el estudio de colocalización logró demostrar que las MPVSs de tipo PII y PIII se unen a la MB de los tres tipos de vasos sanguíneos de la microvasculatura: capilares, arteriolas y vénulas post-capilares. La unión de las MPVSs a la MB de capilares puede estar asociado al mecanismo de acción hemorrágico, dado que estudios de microscopía intravital [31,46,95] y electrónica [98,100,101] han descrito que la hemorragia inducida por MPVSs se da principalmente en capilares. Por otro lado, los efectos patológicos que puede tener la unión de las MPVSs a la MB de las vénulas post-capilares y arteriolas en la microvasculatura se desconocen. Estudios experimentales con veneno completo de vipéridos han mostrado daño a las arterias en cortes histológicos, incluyendo daño a células endoteliales, trombosis y necrosis de fibras de músculo liso en la túnica media [123–125]. Sin embargo, se desconoce los componentes responsables de estos efectos, y si las MPVSs son capaces de unirse a la MB de venas y arterias de mayor calibre.

Finalmente, el presente estudio demostró altos porcentajes de colocalización de las MPVSs de tipo PII y PIII con el colágeno tipo IV de la MB vascular, en comparación a la MPVS de tipo PI. Sin embargo, debido al límite de resolución de la microscopía de luz, no se puede asegurar que las MPVSs se unen al colágeno tipo IV, ya que la interacción podría ser con otros componentes de la MB que están en estrecho contacto con el colágeno IV. Para responder esta interrogante se debe recurrir a técnicas de microscopía de mayor resolución, como la inmunomicroscopía electrónica.

4.1.2. Comparación de MPVSs de tipo PIII hemorrágica y no hemorrágica

Debido a que existen MPVSs no hemorrágicas de tipo PIII, surge la interrogante si estas toxinas se distribuyen de manera similar que las MPVSs hemorrágicas del mismo tipo, ya que ambas enzimas poseen dominios DC. Al comparar la Basparina, una MPVS de tipo PIII no hemorrágica que presenta actividad coagulante, con cantidades equimolares de la CsH1, se observó un patrón de distribución muy diferente. Mientras la CsH1 se localiza alrededor de la MB vascular como se describió en la sección 4.1.1, no se detectó señal fluorescente en el tejido para la Basparina (Figura 2). La actividad proteolítica sobre gelatina de la Basparina marcada con Alexa 647 se mantuvo alrededor de un 75% en comparación con la toxina sin marcar (Figura 3), además, la actividad coagulante *in vitro* no se vio alterada, lo cual demuestra que luego del marcaje la enzima conserva la actividad proteolítica y coagulante.



Figura 2. Inmunolocalización de dos MPVSs tipo PIII con la membrana basal vascular en músculo cremáster de ratón *ex vivo*. Cantidades equimolares de Basparina (6.4 µg), una MPVS no hemorrágica de tipo PIII, y CsH1 (5 µg), una MPVS hemorrágica de tipo PIII, marcadas con Alexa Fluor 647 (azul), fueron aplicadas durante 15 min en los músculos cremaster aislados de ratón. Los tejidos se fijaron con paraformaldehído al 4% y se realizó una inmunotinción con anticuerpo contra colágeno tipo IV (Abcam ab19808) y anticuerpo secundario marcado con Alexa Fluor 488 (verde). Los tejidos se visualizaron en un microscopio confocal de barrido láser Olympus Fluoview FV1000. Tejidos controles incubados con las toxinas sin marcar no mostraron señal fluorescente. La barra de escala representa 30 µm. Ambas imágenes son representativas para una vénula post-capilar.



Figura 3. Actividad proteolítica sobre gelatina de las MPVS Basparina y CsH1 marcadas con el fluorocromo Alexa 647. A) Se cuantificó la actividad proteolítica sobre gelatina de las MPVS Basparina (5 μg) y CsH1 (5 μg) sin marcar y marcadas con Alexa 647 a diferentes tiempos durante 24 h, por medio del kit comercial EnzChek Gelatinase Assay Kit (Molecular Probes E-12055). Los resultados muestran el promedio de tres repeticiones. U.I.F.: Unidades de Intensidad Fluorescente. B) Los resultados muestran el porcentaje de actividad proteolítica sobre gelatina para cada enzima marcada con el fluorocromo Alexa 647 con respecto a la enzima sin marcar.

Esta es la primera vez que se estudia la unión de una MPVS de tipo PIII no hemorrágica con componentes de la MB en modelos que utilizan tejido completo. Los resultados demuestran que la Basparina no es capaz de unirse a componentes de la MB vascular, lo cual sugiere que la Basparina carece de las secuencias presentes en los exositios de las MPVSs tipo PII y PIII hemorrágicas que median la unión con componentes de la MB vascular, o bien que los patrones de glicosilación de la Basparina afectan su capacidad de unión a la MB. La falta de capacidad de unión de la Basparina a la MB puede estar asociado con el hecho de que esta MPVS carece de actividad hemorrágica. Sin embargo, existen MPVSs de tipo PI hemorrágicas que no son capaces de unirse a componentes de la MB [41,42,56], por lo que la unión a la MB no sería el único determinante para la actividad hemorrágica.

4.2. Efectos de MPVSs hemorrágicas tipo PI y tipo PIII en la microvasculatura del músculo cremaster

Los estudios de distribución de las MPVSs hemorrágicas en la microvascultura indicaron claras diferencias entre las de tipo PI con respecto a las de tipo PII y PIII, ya que estas últimas colocalizan con componentes de la MB, mientras que las de tipo PI se distribuyen de manera más difusa en el tejido. Esta diferencia en la distribución podría hacer que las MPVSs tengan diferentes efectos sobre la microvasculatura. Por lo tanto, se decidió estudiar el efecto de la BaP1, una MPVS de tipo PI, y la CsH1, una MPVS de tipo PIII, sobre los tres principales componentes de la microvasculatura (i.e. membrana basal, células endoteliales y células de músculo liso/pericitos) en el músculo cremáster de ratón. Inicialmante se evaluó el efecto dosis y tiempo dependiente de la BaP1 sobre los diferentes componentes de la microvasculatura en el modelo que utiliza el músculo cremáster aislado (i.e. modelo *ex vivo*). De acuerdo con estos resultados se escogió una dosis y tiempo de exposición para estudiar los efectos de la BaP1 en el modelo *in vivo* y compararlo con los efectos de la CsH1 en cantidades equi-hemorrágicas en ambos modelos. La Tabla 1 muestra un resumen de los resultados que se discutirán en las secciones *4.2.1, 4.2.2 y 4.2.3.*

Componentes de la microvasculatura		BaP1 (MPVS tipo PI)					CsH1 (MPVS tipo PIII)						
		Modelo ex vivo			Modelo in vivo			Modelo ex vivo			Modelo in vivo		
		Cap	VPC	Art	Cap	VPC	Art	Cap	VPC	Art	Cap	VPC	Art
Membrana basal	Colágeno IV (intensidad fluorescente)	Ţ		-	Ļ	Ļ	ł	1	4	1'	Ļ	1 ¹	1
Músculo liso/ pericitos	Tamaño hendiduras	NA	t	t	NA			NA		-	NA		
	Densidad hendiduras	NA		1	NA	-		NA	-	-	NA		-
Células endoteliales	VE Cadherina (relocalización)	-		-	-	+		-	-		14	-	

 Tabla 1. Principales efectos de las MPVSs BaP1 y CsH1 sobre los diferentes componentes

 de la microvasculatura en el músculo cremáster murino.

¹La disminución no fue estadísticamente significativa con respecto al control; sin embargo, hay una tendencia a la disminución. Cap: capilares; VPC: vénulas post-capilares; Art: arteriolas, NA: no aplica; (-): sin cambio con respecto al control; (1): disminución con respecto al control; (1): aumento con respecto al control; (+): presencia del efecto.

4.2.1. Efectos sobre componentes de la membrana basal

En el mecanismo de acción de las MPVSs hemorrágicas propuesto por Gutiérrez *et al.* [13] la proteólisis inicial de componentes de la MB por parte de esta toxinas juega un papel importante en el debilitamiento de la estructura del capilar, con la consecuente ruptura y extravasación. Dadas las diferencias en la distribución de las MPVSs de tipo PI y PIII, y la localización de estas últimas con componentes de la MB, resulta de interés estudiar si existe degradación de estos componentes en la microvasculatura del músculo cremáster y si la degradación es diferente entre los dos tipos de MPVS.

En el modelo *ex vivo*, es decir, en ausencia de flujo sanguíneo y aplicación tópica de la toxina en el tejido aislado, la BaP1 disminuyó significativamente la intensidad fluorescente del inmunomarcaje para el colágeno tipo IV de la MB de los capilares, a diferentes dosis y tiempos de exposición, en comparación a los controles (Figura 1B, artículo II). En el caso de las arteriolas (Figura 1A, artículo II) y las VPC (Figura 1C, artículo II), no se encontraron diferencias significativas; sin embargo, hay una tendencia a la disminución para las VPC. Cuando se estudió el efecto de la BaP1 sobre otros componentes de la MB en el modelo *ex vivo*, se encontró que la BaP1 disminuyó la intensidad fluorescente del inmunomarcaje para la laminina en capilares y VPC, pero no en arteriolas (Figura 2A, artículo II). Por otro lado, no se observó ningún efecto en la intensidad fluorescente del inmunomarcaje para el nidogén (Figura 2B, artículo II).

En el modelo *in vivo*, es decir. en presencia de flujo sanguíneo y aplicación intraescrotal de la toxina en el animal anestesiado, el efecto de la BaP1 sobre la disminución en la intensidad fluorescente del inmunomarcaje para el colágeno tipo IV se dio en la MB de los tres tipos de vasos sanguíneos (capilares, arteriolas y VPC) (Figura 3B, artículo II), a diferencia del efecto observado en el modelo *ex vivo* (Figura 3B, artículo II), el cual fue principalmente sobre capilares. Por otro lado, la CsH1 disminuyó la intensidad fluorescente del inmunomarcaje para el colágeno tipo IV de la MB de capilares, VPC y arteriolas, tanto en ausencia como en presencia de flujo sanguíneo (Figura 3C y 3D, artículo II); aunque en algunos casos la disminución no fue estadísticamente significativa, hay una tendencia a la disminución para los tres tipos de vasos sanguíneos.

Los resultados mostraron una disminución de la inmunotinción para el colágeno tipo IV y la laminina de la MB de los vasos sanguíneos, lo que puede sugerir una degradación de estos componentes por parte de las MPVSs. Estudios previos han demostrado la capacidad de estas y otras MPVSs de degradar el colágeno tipo IV, laminina y nidogén en diferentes modelos *in vivo* [41,107–109,111]. Sin embargo, estos estudios utilizan homogenizados de tejidos o exudado inflamatorio, por lo que se desconoce el origen de los productos de degradación detectados. Por lo tanto, los estudios de inmunohistoquímica son necesarios para identificar si la degradación de estos componentes se está dando en la MB vascular. Aunque existen algunos estudios de inmunohistoquímica de cortes de gastronemio [107,109] y piel [41,42,108], éstos muestran una disminución cualitativa de la inmunotinción para colágeno IV y laminina de la MB de cortes transversales de vasos sanguíneos. El estudio de

inmunohistoquímica en el músculo cremáster completo permitió realizar un análisis más detallado y cuantitativo de la degradación de estos componentes en imágenes longitudinales de vasos sanguíneos, así como realizar un análisis comparativo entre las dos MPVSs, el tipo de vaso (i.e. capilar, arteriola, VPC) y la presencia o no de flujo sanguíneo.

La capacidad de las MPVSs de degradar el colágeno tipo IV principalmente en la MB de capilares, tanto en presencia como en ausencia de flujo sanguíneo, da soporte a la hipótesis de que la degradación de este componente puede ser clave en el mecanismo de acción hemorrágico de las MPVSs [41,42,109]. Por otro lado, el hecho de que la degradación del colágeno IV se puede dar en ausencia de flujo sanguíneo apoya el mecanismo de acción en dos pasos propuesto para las MPVSs hemorrágicas, en donde inicialmente se da una degradación de componentes de la MB, el cual puede ocurrir en ausencia de flujo sanguíneo; en el segundo paso se da una distención y ruptura de las paredes de los capilares debido a las fuerzas hemodinámicas normales, lo que provoca extravasación de eritrocitos y otros componentes sanguíneos [13]. Esto coincide además con el importante papel del colágeno tipo IV en la estabilidad mecánica de la MB, ya que es la única proteína de la MB que forma una red unida covalentemente [83–88].

La BaP1 también fue capaz de degradar la laminina de la MB de capilares y VPC en ausencia de flujo sanguíneo. La aparente mayor capacidad de la BaP1 de disminuir la inmunotinción para la laminina en comparación al colágeno tipo IV de la MB de VPC puede ser debido a que el colágeno IV forma una red unida covalentemente [87,88], lo cual hace que sea más difícil perder epítopos del colágeno IV en comparación a la laminina. Por el contrario, estudios previos de inmunohistoquímica de tejido de piel han mostrado un mayor efecto de MPVSs hemorrágicas sobre la degradación de colágeno IV en comparación a laminina [41,42]. Esta discrepancia puede deberse a que los resultados para la laminina provienen del modelo *ex vivo*, es decir, en ausencia de flujo sanguíneo, mientras que los otros estudios [41,42] utilizan modelos en que la toxina se aplica en presencia de flujo sanguíneo, lo cual puede favorecer la difusión de productos de degradación del colágeno IV.

Por otro lado, la BaP1 no disminuyó la inmunotinción para nidogén, lo que podría indicar que la BaP1 no es capaz de degradar el nidogén de la MB vascular en el músculo cremáster aislado. Sin embargo, estudios previos que utilizan homogenizados de tejidos y Matrigel[®], una preparación de MB de sarcoma de ratón, han demostrado que las MPVSs hemorrágicas son capaces de degradar nidogén [107,109,111]. Esta discrepancia puede deberse a que los productos de degradación de nidogén detectados en los homogenizados de tejidos y que se esté dando la degradación del nidogén, pero que los epítopos permanezcan unidos a la MB, lo cual puede ser detectado por análisis inmunoquímico del homogenizado del tejido, pero no por inmunohistoquímica.

Al comparar el efecto de la BaP1 con la CsH1, en cantidades equihemorrágicas, se encontró que la MPVS de tipo PIII tuvo un mayor efecto en la degradación del colágeno IV de la MB vascular, en comparación a la de tipo PI. Esto puede estar asociado con la diferencia en la distribución de estas dos toxinas en la microvasculatura. La presencia de exositios en los dominios adicionales tipo disintegrina y rico en cisteína en las MPVSs de tipo PIII podrían dirigir a la toxina hacia blancos relevantes en la microvasculatura y degradar componentes específicos de la MB vascular en mayor medida que en caso de las MPVSs de tipo PI.

Al comparar el efecto de las MPVSs sobre la MB entre los diferentes tipos de vasos sanguíneos, se encontró que ambas MPVSs degradaron el colágeno tipo IV de la MB de capilares en mayor medida que en arteriolas y VPC. Por otro lado, la MB de las VPC fue más susceptible a degradación que la MB de arteriolas. En el modelo utilizado, la acción de las MPVSs sobre la MB vascular depende de la accesibilidad a dicha estructura, de la capacidad proteolítica de las MPVSs y de la capacidad de difusión de los fragmentos degradados y pérdida de epítopos después de la proteólisis. Los estudios de colocalización demostraron que la unión a la MB es similar entre los diferentes tipos de vaso para la MPVS de tipo PIII. Por otro lado, la BaP1 fue capaz de degradar la laminina en VPC, lo cual demuestra que la BaP1 es capaz de llegar a dicha estructura. Por lo tanto, se descarta que la menor degradación del colágeno tipo IV de la MB observado en arteriolas y VPC se deba a

una menor accesibilidad de las toxinas a dicha estructura. Por lo consiguiente, estas diferencias podrían deberse a una mayor facilidad de difusión de los fragmentos degradados desde la MB de capilares, dado que estos vasos están conformados solamente por una capa de células endoteliales y una MB circundante [60].

En el mecanismo de acción hemorrágico de las MPVSs propuesto por Gutiérrez et al. [13], las fuerzas hemodinámicas normales (i.e. presión hidrostática y la fuerza de cizalla) juegan un papel importante en la distensión y ruptura de las células endoteliales, luego de la degradación de componentes de la MB por parte de las MPVSs. Por esta razón, se decidió estudiar el efecto de las MPVSs en presencia y ausencia de flujo sanguíneo. Estudios previos han mostrado que la BaP1 disminuye el grosor y afecta la integridad de la MB de capilares [112], además reduce la densidad de capilares en cortes histológicos de gastronentio de ratón en presencia de flujo sanguíneo [126]; sin embargo, en condiciones en donde se interrumpe el flujo sanguíneo estos efectos no se observan. Estos hallazgos no descartan la posibilidad de que componentes de la MB vascular sean hidrolizados aún en asuencia de flujo sanguíneo, lo cual no podría evidenciarse en estudios de microscopía electrónica. En el presente estudio fue posible realizar un análisis cuantitativo de la intensidad fluorescente en imágenes longitudinales de vasos sanguíneos, lo cual demostró que ambas MPVSs son capaces de degradar componentes de la MB de capilares tanto en presencia como en ausencia de flujo sanguíneo.

Por otro lado, cuando se comparó el efecto de las MPVSs sobre la MB vascular en presencia y ausencia de flujo sanguíneo, se encontró que la CsH1 disminuyó la inmunotinción para el colágeno tipo IV de la MB de los tres tipos de vasos sanguíneos de manera similar en condiciones con y sin flujo sanguíneo. En el caso de la BaP1, el efecto sobre el colágeno tipo IV se extendió a la MB de arteriolas y VPC en condiciones de flujo sanguíneo, mientras que el efecto sobre la MB de capilares se observó de manera similar que en el modelo *ex vivo*. En otros términos, para la MPVS de tipo PIII el efecto sobre la MB vascular fue similar en presencia y ausencia de flujo sanguíneo, mientras que para la MPVS de tipo PI el efecto fue mayor en condiciones de flujo sanguíneo. Este diferente efecto para la BaP1 puede deberse a la distribución de las toxinas dadas las diferentes condiciones

experimentales. En el modelo *ex vivo*, la BaP1 se aplican de manera tópica en el tejido aislado, a temperatura ambiente, en ausencia de flujo sanguíneo y linfático; bajo estas condiciones la MPVS podría tener una menor difusión. Por el contrario, en el modelo *in vivo*, el flujo sanguíneo y linfático, además de la temperatura corporal, pueden aumentar la difusión de la BaP1 y permitir que alcance la MB de arteriolas y VPC en mayor concentración. Además, en condiciones *in vivo* puede haber una mayor capacidad de difusión de los fragmentos degradados a través del drenaje linfático. Por otro lado, se ha descrito que ante un aumento de las fuerzas hemodinámicas que operan bajo condiciones de flujo sanguíneo, la MB puede ser más suceptible a la acción de proteinasas [127–130]; sin embargo, esta hipótesis debe ser estudiada con mayor detalle. En el caso de la CsH1, la capacidad de unión y afinidad a componentes de la MB permite que la toxina alcance la MB de los tres tipos de vasos sanguíneos de manera similar, tanto en el modelo *ex vivo* como en el *in vivo*.

4.2.2. Efectos sobre células endoteliales

Las células endoteliales, junto con la MB, conforman la principal barrera de permeabilidad vascular [62,63]. Estudios previos en modelos *in vivo* han mostrado alteraciones en las células endoteliales en los primeros minutos después de la aplicación de una MPVS hemorrágica, efecto asociado con la distensión y ruptura de capilares como consecuencia de la proteólisis de componentes de la MB [99,101], y no por un efecto citotóxico directo [57,58,95]. Por otro lado, se ha descrito la hemorragia *per diapédesis* como un posible mecanismo de las MPVSs hemorrágicas, el cual consiste en extravasación de eritrocitos a través de las uniones entre las células endoteliales de vénulas debido al proceso inflamatorio que se genera [96,97]. Por esta razón, es de interés estudiar el efecto de las MPVSs sobre las células endoteliales en el modelo de estudio y su actividad sobre la permeabilidad vascular *in vivo*.

Para evaluar el efecto de las MPVSs sobre el endotelio vascular se utilizó un anticuerpo contra la VE cadherina para visualizar la unión entre las células endoteliales en los vasos sanguíneos de la microvasculatura del músculo cremáster. En el modelo *ex vivo*,

ninguna de las dos MPVSs hemorrágicas estudiadas provocó cambios en la intensidad fluorescente total (Figura 4), ni en la estructura y organización de la unión endotelial vascular, con respecto al control. Sin embargo, en el modelo *in vivo*, con la BaP1 se observó interrupciones en la continuidad del marcaje para la VE cadherina en el 50% de las imágenes de VPC analizadas, sin cambios en la intensidad fluorescente total, en comparación al control (Figura 4, artículo II). Este cambio en la organización de la VE cadherina en VPC no se observó para la CsH1; tampoco se observó en capilares ni arteriolas para ninguna de las dos MPVSs.



Figura 4. Efecto de la A) BaP1 y B) CsH1 sobre la intensidad fluorescente total para el inmunomarcaje de la VE Cadherina en la microvasculatura del músculo cremáster de ratón. Los músculos cremáster aislados se incubaron con 30 μ g de BaP1 o 15 μ g de CsH1 (modelo sin flujo sanguíneo). En otro experimento, los ratones anestesiados se inyectaron por vía intraescrotal con 60 μ g de BaP1 o 30 μ g de CsH1 (modelo con flujo sanguíneo). Los controles se incubaron o se inyectaron con PBS. Después de 15 minutos de exposición a la toxina en cada modelo, se fijaron los músculos cremáster enteros y se inmunomarcaron con el anticuerpo anti-VE cadherina para observación mediante microscopía confocal y análisis de la intensidad fluorescente total. Los resultados se expresan como la media ± SEM del porcentaje de intensidad relacionado con el control de al menos cinco imágenes de cada tipo de vaso por animal (n = 4). La línea punteada horizontal representa el 100% de intensidad fluorescente de los controles.

Por otro lado, para evaluar la permeabilidad vascular inducida por las MPVSs, se midió la extravasación de plasma en piel luego de la aplicación intradémica de las toxinas, utilizando el colorante azul de Evans como marcador. Las MPVSs se compararon en cantidades proporcionales a las utilizadas en los experimentos de inmunohistoquímica. Los resultados mostraron que la BaP1 fue capaz de aumentar la permeabilidad vascular con un área de extravasación de $87 \pm 13 \text{ mm}^2$ (Figura 5, artículo II). Por el contrario, la CsH1 no aumentó la permeabilidad vascular y la mayoría de los tejidos se observaron similares a los controles, con algunas pequeñas áreas de extravasación en algunos animales. Los controles no mostraron áreas de extravasación en la piel.

La VE cadherina es una proteína transmembrana que se expresa exclusivamente en las células endoteliales y tiene funciones importantes en la integridad de los vasos sanguíneos [64,65] y en la regulación de la permeabilidad vascular [66,67]. Los resultados muestran que la BaP1 es capaz de alterar la organización de la unión endotelial en las VPC en el modelo in vivo; además, es capaz de aumentar la permeabilidad vascular luego de la administración intradérmica. La pérdida en la continuidad del marcaje para la VE cadherina no puede deberse a proteólisis sobre dicha proteína, dado que en el modelo ex vivo no se observó el mismo efecto; además, el efecto se presentó solamente en VPC y no en otros vasos sanguíneos, a los cuales también tendría acceso la MPVS. Por otro lado, este efecto inducido por la BaP1 es similar al descrito después de un estímulo inflamatorio en VPC, y está asociado a un aumento de la permeabilidad vascular luego de cambios en la localización, internalización y/o desensamblaje de la VE cadherina [67,131,132]. La BaP1 ha sido ampliamente estudiada por sus propiedades proinflamatorias, formación de edema y aumento de la permeabilidad vascular [22,51,133]. Por lo tanto, el efecto observado con la BaP1 puede deberse a una relocalización de la VE cadherina con un aumento de la permeabilidad vascular como parte de la respuesta inflamatoria que se genera en el tejido, lo cual puede ocurrir bajo las condiciones del modelo in vivo, y no en el modelo ex vivo en ausencia de flujo sanguíneo. En el caso de la CsH1, no se observó el mismo efecto sobre la VE cadherina; tampoco se observó un aumento en la permeabilidad vascular, lo cual sugiere

que esta MPVS posee menor actividad proinflamatoria que la BaP1. Esta menor actividad proinflamatoria podría explicar la diferencia en el efecto de estas dos MPVSs sobre la VE cadherina.

Recientemente, un estudio que utiliza cultivo de células endoteliales demostró la capacidad de una MPVS de tipo PIII de alterar la unión célula-célula por relocalización de la VE cadherina y la y-catetina luego de la proteólisis del receptor LRP5/6, sin degradación de la VE cadherina [59]. En este mismo estudio, el uso de un anticuerpo contra el sitio de corte en el receptor LRP6 disminuyó la hemorragia inducida por la toxina en un modelo in vivo; sin embargo, no se logró demostrar una supresión por completo de la hemorragia [59]. Estos resultados sugieren que las MPVSs podrían favorecer la salida de eritrocitos al intersticio por un aumento de la permeabilidad vascular luego de la proteólisis del receptor LRP5/6 y reubicación de la VE cadherina. El efecto de la BaP1 sobre la VE cadherina y el aumento en la permeabilidad vascular podría estar asociado con este mecanismo descrito en la literatura, lo cual coincide y da soporte al mecanismo de hemorragia per diapédesis propuesto previamente [96,97]. Sin embargo, el hecho de que la CsH1 a dosis equihemorrágicas no aumentó la permeabilidad vascular ni tuvo efecto sobre la VE cadherina, y que el anticuerpo contra el receptor LRP6 no suprimió por completo la hemorragia [59], sugiere que existen otros mecanismos como la hemorragia per rhexis [13,33,44], y que la hemorragia per diapédesis asociada con la apertura de uniones intercelulares en endotelio venular por sí solo no es suficiente para inducir hemorragia, aunque puede contribuir a la hemorragia de las MPVSs con potente actividad proinflamatoria.

4.2.3. Efectos sobre pericitos y células de músculo liso

Las células de músculo liso y pericitos son otros componentes importantes de la microvasculatura. Las arteriolas poseen una capa de células de músculo liso con uniones estrechas entre las células adyacentes [61]. Por otro lado, las VPC poseen una menor cantidad de células de músculo liso, pero poseen células denominadas pericitos, los cuales son un tipo de célula de músculo liso especializado que forma una red de células embebidas dentro de la MB formando hendiduras u orificios entre las células adyacentes [60]. Dado que

los estudios de colocalización y degradación de componentes de la MB demostraron que las MPVSs son capaces de alcanzar la MB de arteriolas y VPC, y que los efectos de las MPVSs sobre células de músculo liso y pericitos no han sido estudiados anteriormente, surge el interés de evaluar el efecto de estas toxinas sobre dichos componentes en el modelo de estudio.

En el modelo *ex vivo*, la BaP1 provocó en las arteriolas un incremento en el tamaño y la densidad de las hendiduras entre las células del músculo liso adyacentes, en comparación a los controles (Figura 6A, 6C, artículo II). Por otro lado, la BaP1 provocó en las VPC un aumento en el tamaño, pero no en la densidad, de las hendiduras entre las células del músculo liso y pericitos adyacentes, en comparación a los controles (Figura 6, artículo II). En otros términos, la BaP1 creó nuevas hendiduras en las arteriolas, mientras que aumentó el tamaño de las hendiduras existentes en las VPC, en condiciones *ex vivo*. Este efecto sobre las hendiduras entre las células de músculo liso y pericitos no se observó cuando la BaP1 se evaluó en el modelo *in vivo*, y tampoco se observó para la CsH1 en ninguno de los dos modelos.

Estudios previos han demostrado un incremento en las hendiduras de las VPC luego de un estímulo inflamatorio [71,72,134]. Sin embargo, el incremento en el tamaño de las hendiduras en las VPC y formación de nuevas hendiduras en las arteriolas provocado por la BaP1 solo se observó en condiciones *ex vivo*, lo cual se descarta que el fenómeno observado pueda deberse a una respuesta inflamatoria. Por otro lado, en condiciones de isquemia se ha descrito que los pericitos y células del músculo liso vascular pueden sufrir contracción y daño celular [135–137]. En el modelo de músculo cremáster aislado la condición de isquemia puede provocar la contracción y daño de estas células, lo cual podría ser potenciado por la presencia de la BaP1, efecto que puede prevenirse en condiciones de flujo sanguíneo. En el caso de la CsH1, no se observó el mismo efecto que con la BaP1, lo cual podría explicarse por la presencia de exositios en los dominios adicionales de la CsH1, los cuales puede dirigir y concentrar la toxina hacia blancos de la MB y no en músculo liso y pericitos. Por el contrario, la BaP1 puede actuar sobre diferentes estructuras en el tejido debido a que

carece de una unión específica a componentes de la microvasculatura, como fue demostrado en los estudios de distribución y colocalización.

Los pericitos son células que están en estrecho contacto con las células endoteliales y tienen funciones importantes en el mantenimiento de la integridad vascular, contracción vascular, regulación del flujo sanguíneo, remodelamiento de la MB durante el proceso inflamatorio, angiogénesis, y cicatrización de heridas [71-77]. Por lo tanto, el efecto que puedan tener las MPVSs sobre estas células y las células de músculo liso puede jugar un papel importante en la fisiopatología del daño tisular inducido por los venenos de los vipéridos. Estudios previos han demostrado que el veneno de B. asper puede afectar las células de músculo liso de los vasos linfáticos por acción de una PLA₂ [138]. Otros estudios con veneno completo de vipéridos han mostrado daño en las arterias en cortes histológicos, incluyendo necrosis de fibras de músculo liso en la túnica media [123-125]. Sin embargo, la acción sobre las células de músculo liso vascular y pericitos observada en el presente estudio no ha sido descrito para alguna MPVS. Esto es un aspecto que debe ser estudiado a profundidad, principalmente en el contexto del accidente ofídico, ya que las MPVSs, especialmente las de tipo PI, en conjunto con las PLA₂ podrían afectar las células de músculo liso y pericitos de arteriolas y vénulas con consecuencias en los procesos de daño y regeneración del tejido.

4.3. Degradación de componentes de la membrana basal y matriz extracelular

Debido a la importancia que tiene la degradación de componentes de la MB vascular en el mecanismo de acción hemorrágico de las MPVSs propuesto por Gutiérrez *et al.* [13], se decidió profundizar en el estudio de la degradación de componentes de la MB y de la MEC circundante, tanto con las MPVSs aisladas como con veneno completo de *B. asper*, por medio de modelos *in vivo* que utilizan herramientas como la proteómica y la inmunodetección por western blot en homogenizados de piel y exudado inflamatorio. Estas herramientas fueron utilizadas en un estudio anterior que comparó dos MPVSs de tipo PI, una hemorrágica y otra no hemorrágica, en modelos *in vivo*, lo cual brindó información importante en cuanto al mecanismo de acción de estas toxinas [109]. El presente estudio compara por primera vez tres MPVSs hemorrágicas de tipo PI, PII y PIII utilizando estas mismas herramientas y modelos.

4.3.1. Degradación por parte de MPVSs hemorrágicas de tipo PI, PII y PIII

Al administrar 75 µg de BaP1, 1.5 µg de BlatH1 y 35 µg de CsH1 por vía intradérmica en la región ventral abdominal de los ratones, se observaron lesiones hemorrágicas de tamaño e intensidad similar luego de 15 minutos de la administración, lo cual confirma que las cantidades administradas son equihemorrágicas. De estas lesiones hemorrágicas, se tomó secciones de piel de igual tamaño y se preparó una mezcla de homogenizado de tejido por tratamiento, para la inmunodetección de diferentes componentes de la MB y MEC. El patrón de bandas detectadas en los homogenizados de los tejidos tratados con las MPVSs se comparó con el patrón de bandas detectadas en los tejidos control, para cada proteína estudiada (Figura 3, artículo I). La tabla 2 muestra la masa molecular de las bandas detectadas por western blot en los homogenizados de piel control y el cambio que ocurre luego de la administración de las MPVSs estudiadas, así como la aparición de nuevas bandas. La disminución en la intensidad de bandas con respecto al control puede deberse a la acción proteolítica de las MPVSs sobre los componentes detectados; por otra parte, el aumento en la intensidad de bandas, así como la aparición de nuevas bandas, puede deberse a la aparición de productos de degradación debido a la proteólisis sobre dichos componentes.

Tabla 2. Masa molecular (MM) de las bandas detectadas por western blot para componentes de la MB y MEC circundante en los homogenizados de piel control y el cambio que provocan las MPVSs.

	MM de bandas	Cam	bio con respecto a co	ontrol
	en control (kDa)	CsH1 (PIII)	BlatH1 (PII)	BaP1 (PI)
	216	-	-	
	176	-		-
Colágeno IV	165	-	-	-
	117	=	-	.04
	107*	1	L.	1
	97	i	TT I	11
	216	=	-	1
	160	-		tt
	140	-	-	=
Colágeno VI	120	-		=
-	110	-	-	=
	53	-	-	11
	Nuevas bandas	192 y 25	192 y 25	192 y 25
	350	=	10	
	270	-	=	-
Laminina	167*	-		=
	140	-	-	tt
	Nuevas bandas			225 y 50
	135*	=	-	11
	120	11	11	11
Nidogén 1	70	11	11	11
	47	=	tt	1
	Nuevas bandas	100		

Banda predominante. (1) Disminución de dos a cinco veces con respecto al control. (11) Disminución más de cinco veces con respecto al control. (1) Aumento de dos a cinco veces con respecto al control. (1) Aumento más de cinco veces con respecto al control. (=) No hay cambio con respecto al control.

Con respecto a la inmunodetección para el colágeno IV, se observó que las tres MPVSs provocaron un patrón de degradación muy similar, donde la banda predominante de 107 kDa disminuyó considerablemente con respecto al control, y hubo un aumento del producto de degradación de 97 kDa (Figura 3A, artículo I). La intensidad de la banda de 97

KDa fue mayor para la BaP1, seguido de la BlatH1 y la CsH1. En el caso del colágeno tipo VI, las tres MPVSs generaron productos de degradación de 192 y 25 kDa (Figura 3B, artículo I). Adicionalmente, la BaP1 provocó una reducción de la banda predominante de 216 kDa y la banda de 53 kDa, junto con un aumento de la banda de 160 kDa, efectos no observados para las MPVSs PII y PIII. En cuanto a la inmunodetección de laminina, la BaP1 fue la única que provocó cambios en el patrón de bandas, con un aumento de la banda de 140 kDa y la aparición de productos de degradación de 250 y 50 kDa. Finalmente, el patrón de degradación del nidogén 1 fue muy diferente entre las tres MPVSs, donde la BaP1 mostró mayor actividad proteolítica ya que provocó la desaparición de las bandas de 135, 120 y 70 kDa; mientras que la CsH1 y BlatH1 no afectaron la banda predominante de 135 kDa, pero sí la de 120 y 70 kDa. Además, la BaP1 y BlatH1 aumentaron la banda de 47 kDa, efecto que no se observó con la CsH1. En términos generales, se puede observar que el patrón de degradación para el colágeno IV fue similar entre las tres MPVSs, mientras que los patrones de degradación para el colágeno VI, laminina y nidogén son muy distintos, donde la BaP1 presentó una mayor degradación de estos tres componentes.

Las bandas detectadas en los homogenizados de piel luego de la administración de las MPVSs corresponden a proteínas que, aunque sean hidrolizadas, permanecen de alguna manera en el tejido. Sin embargo, puede haber productos de degradación o proteínas completas que son removidas del tejido debido a la acción de las MPVSs y al daño tisular, por lo que pueden aparecer en el exudado inflamatorio. Es por esta razón que se decidió detectar componentes de MB y MEC en el exudado inflamatorio inducido por las MPVSs, 15 min luego de la administración IM en el gastronemio de ratón, por medio de inmunodetección por western blot y análisis de proteómica. En este caso no es posible contar con un control de referencia pues el vehículo en que se disuelven las toxinas no es capaz de provocar una respuesta inflamatoria, y por ende no es posible obtener un exudado, por lo que la comparación se realiza entre las MPVSs en cantidades equihemorrágicas.

El análisis de inmunodetección por western blot de los exudados inflamatorios revela que las MPVSs son capaces de generar productos de degradación, o bien liberar proteínas completas o subunidades, provenientes del colágeno IV, colágeno VI, laminina y nidogén 1 (Figura 4, artículo I). La tabla 3 muestra la masa molecular de las bandas detectadas en el exudado inflamatorio inducido por las MPVSs. El patrón de bandas para el colágeno IV es similar entre las tres MPVSs, con una banda predominante de 90 kDa. Esta banda no coincide con la masa molecular de las cadenas alfa del colágeno IV (145-160 kDa); por lo tanto, esta banda corresponde a un producto de degradación. En el caso de la inmunodetección del colágeno VI, laminina y nidogén 1, el patrón de bandas es muy diferente entre las tres MPVSs. En el exudado inducido por la BaP1, se detectó una menor cantidad y/o intensidad de bandas para el colágeno VI, laminina y nidogén 1.

	MM de las bandas detectadas en el exudado (kDa)				
	CsH1 (PIII)	BlatH1 (PII)	BaP1 (PI)		
	140				
Colágeno IV	90	90	90		
		20	20		
	225		225		
		220			
	200	200			
Colágeno VI	160				
			150		
	140				
	100		100		
	40				
	275	275	275		
			230		
			200		
Laminina	190	190			
			150		
	140				
			105		
	50		50		
Nidogén 1		140			
-	50	50			

 Tabla 3. Masa molecular (MM) de las bandas detectadas por western blot para componentes

 de la MB y MEC circundante en el exudado inflamatorio inducido por las MPVSs.

Por otro lado, el análisis de proteómica permitió la identificación de un total de 297, 354 y 322 proteínas en los exudados inflamatorios inducidos por las MPVSs de tipo PI, PII y PIII, respectivamente (Tabla S1, artículo I). Dentro de estas proteínas se encuentran proteínas séricas (Tablas S2-S4, artículo I), proteínas intracelulares (Tablas S5-S6, artículo I), proteínas asociadas a membranas (Tabla S7, artículo I) y proteínas de MEC (Tabla 2, artículo I). Aunque existen diferencias en el valor cuantitativo de algunas proteínas en los diferentes grupos expuestos anteriormente, el presente trabajo se enfoca en las proteínas de la MEC. Es importante mencionar que se obtuvieron valores cuantitativos similares de cadenas de hemoglobina en los exudados inducidos por cada MPVSs, lo cual confirma que la administración de estas toxinas se realizó en cantidades equihemorrágicas. Con respecto a las proteínas de MEC, se identificaron en total 12 proteínas en el exudado inducido por las MPVSs de tipo PI y PIII, y 9 proteínas en el exudado inducido por la MPVS de tipo PII (Tabla 2, artículo I). La mayoría de estas proteínas corresponden a fragmentos de degradación de acuerdo con el rango de masa molecular en donde fueron detectadas en el gel de SDS-PAGE (Tabla 3, artículo I). De los cuatro principales componentes de la MB, solamente se identificó al nidogén 1 y el perlecan en los exudados inducidos por las tres MPVSs, sin diferencias importantes entre las MPVSs en el valor cuantitativo, ni en el porcentaje de degradación. Por otro lado, existe una tendencia a mayores valores cuantitativos de otras proteínas de MEC detectadas en el exudado inducido por la MPVS de tipo PI en comparación a las otras dos MPVSs, tales como tenascina, vitronectina, colágeno VI, colágeno XIV, colágeno III y trombospondina-4.

4.3.2. Degradación por parte del veneno de B. asper en el tiempo

El estudio de las MPVSs aisladas puede brindar información relevante en cuanto al mecanismo de acción de estas toxinas; sin embargo, debido a que el veneno de las serpientes es una mezcla compleja de componentes que pueden funcionar de manera sinérgica, también es importante realizar estudios con el veneno crudo para un mayor entendimiento del fenómeno hemorrágico en el contexto del accidente ofídico. La patología de la hemorragia local inducida por el veneno crudo de *B. asper* ha sido estudiada por medio de metodologías como histología, estudios de ultraestructura, inmunohistoquímica y cuantificación de

marcadores específicos en homogenizados de tejidos o fluidos [8,100,139,140]. Recientemente, un estudio de proteómica del exudado inducido por el veneno de *B. asper* evidenció la capacidad de las MPVSs presentes en el veneno de degradar proteínas de MEC [113]. Este estudio fue realizado en un tiempo temprano del envenenamiento, por lo que surge la necesidad de estudiar la variación en el tiempo de las proteínas de MEC por medio de estudios de proteómica e inmunoquímica del exudado inflamatorio inducido por el veneno de *B. asper*, con el fin de identificar biomarcadores de daño y remodelamiento de la MEC característico de estadíos tempranos y tardíos del envenenamiento ofídico.

El análisis de inmunodetección por western blot de componentes de la MEC presentes en el exudado inflamatorio inducido por el veneno de B. asper revela la presencia de productos de degradación, o bien proteínas completas o subunidades provenientes del colágeno IV, laminina, nidogén 1, colágeno I, colágeno VI y fibronectina, con diferencias en el patrón de bandas, en el transcurso de 24 h de envenenamiento (Figura 3, artículo III). La tabla 4 muestra la masa molecular de las bandas detectadas en el exudado inflamatorio inducido por el veneno de B. asper en diferentes tiempos. Para el colágeno IV, se detectaron bandas de diferentes pesos, con una banda predominante de 107 kDa en las primeras 6 horas de envenenamiento, cuya intensidad disminuye a las 6 h en comparación a la hora. Esta banda predominante corresponde a un producto de degradación ya que no coincide con la masa molecular de las cadenas alfa del colágeno IV (145-160 kDa). Por otro lado, a las 24 h no se detectó ninguna banda para el colágeno IV. En el caso de la laminina, aparecen dos bandas predominantes de 220 y 140 kDa, cuya intensidad incrementa en el tiempo. La masa molecular de estas bandas coincide con la masa molecular de las cadenas alfa, beta y gama que componen la laminina (130-200 kDa). Para el nidogén 1 se detectaron dos bandas predominantes de 120 y 40 kDa, las cuales no coinciden con la masa molecular esperada para el nidogén 1 (137 kDa) por lo que es probable que se traten de productos de degradación. Además, se observó una disminución de la banda de 40 kDa con un aumento de la banda de 120 kDa en el tiempo. Para el colágeno I se detectaron tres bandas predominantes de 135, 120 y 107 kDa en las primeras 6 horas de envenenamiento, las cuales coinciden con la masa molecular de algunas de las cadenas alfa del colágeno I (115 y 120 kDa). La intensidad de estas bandas es mayor a las 6 h en comparación a 1 h, y no se detectó ninguna banda para el colágeno I a las 24 h. En el caso del colágeno tipo VI, se detectó una banda predominante de 230 kDa, la cual aumenta en el tiempo, y una banda menos intensa de 118 kDa, la cual disminuye en el tiempo. Estas bandas coinciden con la masa molecular de algunas de las cadenas alfa que conforman el colágeno VI (110 y 244 kDa). Finalmente, para la fibronectina se detectaron dos bandas predominantes de 265 y 236 kDa, las cuales incrementan en el tiempo, y otras bandas adicionales de menor intensidad. La masa molecular de la banda de 265 kDa coincide con la masa molecular esperada para la fibronectina (262 kDa). En resumen, de acuerdo con los resultados de inmunoblotting, hay una rápida degradación del colágeno IV en las primeras 6 horas, mientras que el colágeno I tiende a aumentar en el exudado a las 6 h en comparación a 1 h, ninguno de estos dos colágenos se detectó a las 24 h; en el caso de laminina, nidogén, colágeno VI y fibronectina, hay una mayor degradación de estas proteínas en las primeras horas y luego aumenta su presencia en el exudado como proteínas completas a las 24 h.

Tabla 4. Masa molecular (MM) de las bandas detectadas por western blot para componentes de la MB y MEC circundante en el exudado inflamatorio inducido por el veneno de *B. asper* en diferentes tiempos.

	MM de las bandas detectadas en el exudado (kDa)			
	1 h	6 h	24 h	
	217	217		
Colágeno IV	172	172		
	135	135		
	107*	107*		
	70	70		
Laminina	220	220	220	
		140	140	
Nidogén 1		120	120	
	40	40		
	135	135		
Colágeno I	120	120		
	107	107		
Colágeno VI	230	230	230	
	118	118		
	265*	265*	265*	
Fibronectina	236*	236*	236	
	175	175	175	
	140	140		
	100	100		

Banda predominante.

Por otro lado, el análisis de proteómica permitió la identificación de un total de 537, 578 y 486 proteínas en los exudados inflamatorios inducidos por el veneno de *B. asper* a la 1, 6 y 24 horas, respectivamente (Tabla S1, artículo III). Dentro de estas proteínas se encuentran proteínas intracelulares (Tablas 1 y S2, artículo III), proteínas asociadas a membranas (Tabla S3, artículo III), proteínas séricas (Tablas S3 y S4, artículo III) y proteínas de MEC (Tabla 2, artículo III). Al igual que en el caso de los exudados inducidos por las MPVSs aisladas, el análisis se enfoca en las proteínas de la MEC. Con respecto a las proteínas de MEC, se identificaron en total 21, 24 y 13 proteínas en el exudado inducido por el veneno de *B. asper* a la 1, 6 y 24 horas, respectivamente. Estas proteínas se organizaron para su análisis en aquellas cuyo valor cuantitativo cambió al menos tres veces en

comparación con otro tiempo y las proteínas que no mostraron cambios significativos entre los tres tiempos (Tabla 2, artículo III). La mayoría de las proteínas de MEC detectadas en el exudado mostraron cambios significativos en su abundancia relativa en el tiempo. Con respecto a las proteínas de la MB, los cuatro componentes principales fueron detectados en los exudados en diferentes tiempos, donde la proteína central del proteoglicano heparán sulfato específico de MB (perlecan), seguido de las cadenas alfa 1 y 2 del colágeno tipo IV, fueron las proteínas de MB más abundantes en los exudados (Figura 2A, artículo III). Estas dos proteínas, junto con el nidogén 2, aparecieron en los exudados en la primera hora, disminuyendo la cantidad con el paso del tiempo, hasta ser indetectables a las 24 h. Por el contrario, la cantidad de laminina y-1 detectada en los exudados aumentó a las 6 y 24 h, y la cantidad de nidogen-1 aumentó a las 6 h en comparación con 1 y 24 h. Con respecto a otros colágenos no fibrilares de la MEC, se detectaron los colágenos VI, XV y XVIII en la primera hora (Figura 2A, artículo III), y su cantidad disminuyó en el tiempo, llegando a ser indetectable a las 24 h. Por otro lado, se detectaron cadenas de los colágenos fibrilares I y III, donde la cantidad de colágeno I aumentó con el tiempo, siendo mayor a las 24 h. Otras proteínas MEC detectadas en los exudados cuya abundancia fue mayor a las 6 h en comparación con 1 y 24 h fueron colágeno III, fibrilina 1 y 2, proteoglicano 4 sulfato de condroitina y colágeno XII. Por otro lado, la trombospondina l apareció en los exudados en la primera hora y la cantidad disminuyó con el tiempo. Otras proteínas de MEC detectadas en los exudados cuyas cantidades no variaron más de tres veces entre los tiempos fueron fibronectina, trombospondina-4, vitronectina, dermatopontin, proteoglicano 4, colágeno tipo XIV y lumican.

4.3.3. Análisis conjunto de la degradación de componentes de la MB y MEC por parte de las MPVSs y el veneno completo de B. asper.

En el presente estudio, las tres MPVSs fueron comparadas en cantidades equihemorrágicas, es decir en cantidades tales que indujeran una lesión hemorrágica similar según la vía de administración. Por lo tanto, se espera que las tres toxinas degraden de manera similar las proteínas de MEC cuya proteólisis es responsable directo de la hemorragia, y, por otro lado, que aparezcan productos de degradación de estas proteínas en

cantidades similares en el exudado. El análisis de inmunoquímica, tanto del homogenizado de piel como del exudado inflamatorio inducido por las tres MPVSs hemorrágicas, revela un patrón de degradación similar para el colágeno IV, a diferencia de la laminina, nidogén y colágeno VI. Con respecto al análisis de proteómica, se identificó a la fibronectina, perlecan, lumican y nidogén 1 como proteínas presentes en cantidades similares en cada exudado. Por otro lado, al comparar los patrones de degradación que provocan las tres MPVSs sobre componentes de la MEC, se puede observar, en términos generales, que la MPVS tipo PI parece degradar en mayor medida al colágeno VI, laminina y nidogén 1. Además, de acuerdo con el análisis de proteómica, la MPVS tipo PI provocó la aparición de mayores cantidades de diversas proteínas de la MEC en los exudados. Esta mayor actividad de la MPVS tipo PI puede deberse a que se inyectó una cantidad molar más alta en comparación a las otras dos MPVSs, debido a su menor actividad hemorrágica, lo cual resultó en una mayor actividad proteolítica en el tejido. Por otro lado, la presencia de exositios en las MPVSs tipo PII y PIII localiza estas enzimas cerca de proteínas específicas de la MEC, reduciendo así la probabilidad de que actúen de manera generalizada sobre otros componentes de la MEC. Por el contrario, la MPVS tipo PI, al carecer de tales exositios, tendría menos restricción para difundirse en los tejidos e hidrolizar una mayor cantidad de componentes de la MEC.

Por otro lado, debido a que la hemorragia aparece en los primeros minutos, se espera que los productos de degradación de proteínas de la MEC cuya proteólisis directa es responsable del daño a la microvasculatura aparezcan en el exudado rápidamente y en mayor cantidad en tiempos tempranos en comparación a tiempos tardíos. La aparición de componentes de la MEC en los exudados puede ser debido a la proteólisis por parte de MPVSs o MMPs endógenas que se producen durante la respuesta inflamatoria. Estudios previos han detectado componentes del veneno de *B. asper* en homogenizados de músculo durante la primera semana de envenenamiento [141]; sin embargo, se sabe que la actividad de las MPVSs se mantiene durante la primera hora y luego disminuye a las 6 y 24 h [142] debido probablemente a la difusión de los componentes del veneno, a la inhibición de las enzimas por parte de inhibidores de proteasas o simplemente por pérdida de actividad enzimática. Por otro lado, se ha visto que las MMPs endógenas, principalmente la MMP-9 y la MMP-2, aparecen en mayor cantidad en tiempos tardíos luego de la administración del veneno completo de *B. asper* o alguna de las MPVSs y PLA₂ aisladas del veneno [49,141,142]. Por lo tanto, la aparición temprana (*i.e.* 1 hora) de proteínas de la MEC en el exudado es debida principalmente a la acción de las MPVSs presentes en el veneno, mientras que la aparición tardía (*i.e.* 24 horas) es debida principalmente a MMPs endógenas que se producen durante la respuesta inflamatoria y forman parte de los procesos de reparación del tejido. Sin embargo, es importante considerar que la presencia de proteínas de la MEC en los exudados también puede ser producto de la liberación de dicho componente como consecuencia del daño en los tejidos sin que medie degradación por parte de proteasas, o también pueden ser proteínas que estén presentes en el plasma y, por lo tanto, pueden salir en el exudado. El análisis de proteómica del exudado inducido por el veneno completo de *B. asper* que se realizó en el presente trabajo detectó productos de degradación del colágeno IV, perlecan, nidogén 2, colágeno VI, colágeno XV y colágeno XVIII en la primera hora, cuya cantidad disminuyó a las 6 h y fue indetectable a las 24 h. Por el contrario, los colágenos fibrilares, aparecen en la primera hora, pero su cantidad aumenta con el tiempo.

A continuación, se va a realizar un análisis de cada uno de los componentes que aparecen en los exudados inducidos tanto por las MPVSs como por el veneno completo. Debido a la importancia que tiene la MB en la estabilidad de la microvasculatura, especialmente para los capilares, el análisis de la degradación de proteínas de la MEC se va a enfocar inicialmente en los cuatro principales componentes de la MB vascular: colágeno IV, laminina, perlecan y nidogén, seguido de un análisis de los otros componentes de la MEC que aparecen en el exudado.

Colágeno IV

El hecho de que el colágeno IV es degradado de manera similar por las tres MPVSs hemorrágicas y que aparece en mayor cantidad en el exudado en la primera hora, apoya el concepto de que esta proteína puede ser un blanco clave de las MPVSs, cuya degradación es un paso importante en el mecanismo de acción hemorrágico de estas toxinas. Además, en el presente estudio se demostró que las MPVSs hemorrágicas son capaces de degradar el colágeno tipo IV de la MB vascular y que existe un alto porcentaje de colocalización con este componente. Esta hipótesis coincide con un estudio previo que identifica al colágeno IV como posible blanco de las MPVSs hemorrágicas, ya que se demostró que una MPVSs hemorrágica degradó en mayor medida este componente en comparación con otra no hemorrágica [109]. Existen otros estudios que han demostrado la capacidad de MPVSs hemorrágicas de degradar el colágeno IV en modelos *in vitro* que utilizan proteína aislada [105,143], fragmentos recombinantes del colágeno IV [144] o matrigel[®] [42,109,111], así como en modelos *in vivo* [41,109]. Por lo tanto, los resultados del presente estudio coinciden con estudios anteriores que han evidenciado la importancia del colágeno IV en el mecanismo de acción hemorrágico de las MPVSs [36,41,42,105,109,111,144]. Además, esta hipótesis está en concordancia con el importante papel que tiene el colágeno IV en la estabilidad de la MB vascular, al ser la única proteína de la MB que forma una red unida covalentemente [79,83–88,145,146]. Por otro lado, se ha descrito que mutaciones en los genes que codifican para el colágeno IV causan alteraciones en la microvasculatura que pueden llevar a accidentes hemorrágicos en cerebro, riñón y pulmón [147–154].

A pesar de haberse detectado productos de degradación del colágeno IV por inmunoquímica, el análisis de proteómica no fue capaz de detectar productos de degradación del colágeno IV en el exudado inducido por las MPVSs hemorrágicas. Estudios anteriores de proteómica tampoco detectaron productos de degradación del colágeno IV en el exudado inducido tanto por MPVSs, como por veneno completo [108,109,113]. Esto puede ser debido a que la tripsina utilizada en los análisis de proteómica puede generar péptidos pesados y modificados del colágeno IV que no son detectados fácilmente en un análisis MS-MS convencional [89]. Esto pone en evidencia la importancia de combinar ambas técnicas, la inmunoquímica y los análisis de proteómica, para una mejor interpretación de los resultados. En el análisis de proteómica del exudado inducido por el veneno completo sí se logró encontrar por primera vez productos de degradación del colágeno IV, lo cual representa un hallazgo importante.

Perlecan

Según el análisis de proteómica, el perlecan fue detectado en el exudado inducido por las tres MPVSs en cantidades similares y se encuentra degradado en un 50%, aproximadamente. Además, el perlecan fue la proteína de MB más abudante detectada en el exudado inducido por el veneno completo en la primera hora y cuya cantidad disminuye al avanzar el tiempo, de igual forma que lo hace el colágeno IV. Estudios previos de proteómica encontraron que la cantidad de perlecan en el exudado inducido por una MPVS hemorrágica es mayor que en el caso del exudado inducido por una MPVS no hemorrágica [109], y que la cantidad disminuye cuando se administra veneno pre-incubado con un inhibidor de metaloproteinasa en comparación al veneno solo [113]. Estos estudios, junto con nuestros hallazgos, sugieren que la degradación del perlecan puede jugar un papel importante en el mecanismo hemorrágico de las MPVSs. Esta hipótesis coincide con el papel estructural del perlecan al conectar y estabilizar las redes de colágeno IV y laminina de la MB [78,79,146,155,156]. El perlecan también interacciona con diversas proteínas de la MEC como nidogén, colágeno XVIII, fibronectina, trombospondina, fibrilinas, entre otras, y participa en la adhesión celular, formación de cartílago, procesos de inflamación, trombosis y angiogénesis [156–158]. Además, mutaciones en el gen que codifica para el perlecan se han asociado con pérdida de la integridad de la MB en diferentes tejidos, incluyendo la microvasculatura de piel y cerebro, lo cual lleva a cuadros hemorrágicos debido a la dilatación y ruptura de microvasos [159-161].

Laminina y nidogén

Con respecto a los otros dos componentes principales de la MB, las tres MPVSs hemorrágicas generaron diferentes patrones de degradación para el nidogén y la laminina, donde la MPVSs tipo PI mostró una mayor degradación de estos sustratos. En el contexto de una lesión hemorrágica similar inducida por las tres MPVSs, este hallazgo sugiere que la proteólisis de estos componentes no está asociada directamente con el mecanismo de acción hemorrágico de las MPVSs. Por otro lado, en el análisis de proteómica del exudado inducido por el veneno completo, solamente el nidogén 2 aparece en mayor cantidad en tiempos

tempranos, mientras que el nidogén 1 y la laminina tienden a aumentar con el tiempo. Dado que el nidogén 2 es más abundante en la MB vascular que el nidogén 1 [162], la liberación temprana de esta proteína puede ser consecuencia del daño a la microvasculatura luego de la proteólisis de otros componentes, como el colágeno IV y el perlecan. Los resultados del presente trabajo parecen indicar que la degradación de laminina y nidogén no juegan un papel principal en el mecanismo de acción hemorrágico de las MPVSs, lo cual coincide con un estudio previo que descartó a ambos componentes como posibles blancos de las MPVSs hemorrágicas [109].

Sin embargo, no debe dejarse de lado el estudio de las implicaciones que puede tener la degradación de estas proteínas en el daño de la microvasculatura y los procesos de reparación de los tejidos. El nidogén es una glucoproteína monomérica que se encuentra en dos isofomas las cuales interaccionan con laminina, colágeno IV, perlecan y fibulina; no obstante, estudios genéticos indican que no es estrictamente requerida para la arquitectura de la MB, por lo que podría tener otras funciones no estructurales [162–164]. Por otro lado, la laminina se encuentra en múltiples isoformas, y es importante en el ensamblaje inicial de la MB durante la organogénesis; además interacciona con integrinas y con componentes de la MB como nidogén y perlecan, con lo cual conecta las células epiteliales con la MB [165– 167]. La laminina es también importante en la unión epidermis-dermis [168,169] y, por lo tanto, su proteólisis puede estar relacionada con la formación de ampollas que provocan los venenos de los vipéridos.

Colágenos no fibrilares

Estudios de proteómica han evidenciado la aparición de colágenos no fibrilares como los tipos VI, XI, XII, XIV, XV, XVI, XVIII y XIX en el exudado inflamatorio inducido tanto por MPVSs como por veneno completo [108,109,113]. La mayoría de estos colágenos participan en la integración de la MB con la MEC circundante; por lo tanto, la proteólisis de estos colágenos por parte de las MPVSs o su liberación como consecuencia del daño tisular puede contribuir al daño de la MB y desorganización de la MEC.

El colágeno VI es un colágeno formador de filamentos en cuenta que tiene una función importante en la estabilidad y organización estructural al conectar componentes de la MB con colágenos fibrilares de la MEC [116,170,171]. Debido a que estudios in vitro han demostrado la capacidad de MPVSs hemorrágicas de unirse y degradar al colágeno tipo VI [37,115], y que una MPVS hemorrágica generó más productos de degradación del colágeno VI en el exudado inflamatorio en comparación con una no hemorrágica [109], surge la hipótesis de que la degradación del colágeno VI podría ser clave o participar en el mecanismo de acción hemorrágico de estas toxinas. Sin embargo, en el presente trabajo se encontró que las tres MPVSs hemorrágicas generaron diferentes patrones de degradación para el colágeno VI y, además, el colágeno VI apareció en mayor cantidad en el exudado inducido por la MPVS tipo PI; lo anterior sugiere que, en el contexto de una lesión hemorrágica similar, la proteólisis de este componente no está asociada directamente con el mecanismo de acción hemorrágico de las MPVSs. En el exudado inducido por el veneno completo, el colágeno VI aparece en tiempos tempranos, y luego tiende a disminuir de igual manera que lo hacen el colágeno IV y el perlecan, lo cual puede estar asociado a una liberación de esta proteína luego del daño a la MB vascular o a una temprana hidrólisis de esta por parte de las MPVS. El colágeno VI es abundante en la MB de las células musculares [170,172,173] y tiene un papel importante en el proceso de regeneración muscular y en la función de las células satélite [174]; además, deficiencias en este colágeno se han asociado con distrofia muscular y otras miopatías [175-178]. Por lo tanto, las implicaciones que puede tener la degradación de este colágeno en la patología de la mionecrosis y en los procesos de regeneración de las fibras musculares es un aspecto que debe estudiarse con mayor profundidad, especialmente en el contexto del envenenamiento ofídico, debido a la sinergia que podría presentarse con PLA2 miotóxicas. Al igual que en el caso de la laminina, el colágeno VI también es importante en la unión epidermis-dermis [179,180] y, por lo tanto, su proteólisis también puede estar relacionada con la formación de ampollas que provocan los venenos de los vipéridos.

Con respecto a otros colágenos no fibrilares, el colágeno XV y XVIII aparecen en el exudado inducido por el veneno completo en tiempos tempranos, y luego tiende a disminuir

en tiempos tardíos. Además, el colágeno XV fue detectado solamente en el exudado inducido por la CsH1. Los colágenos XV y XVIII son proteoglicanos que conectan la MB con componentes de la MEC; además, son productores de sustancias con propiedades antiangiogénicas conocidas como restina y endostatina, respectivamente [156,181]. En un estudio previo de proteómica que comparó el exudado generado por dos MPVSs tipo PI, una hemorrágica y una no hemorrágica, se encontró que la primera generó más productos del colágeno XV en el exudado inflamatorio [109], lo que podría sugerir alguna participación de la degradación de este colágeno en el mecanismo hemorrágico de las MPVSs; sin embargo, en el presente trabajo el colágeno XV solamente apareció en el exudado inducido por la MPVS tipo PIII. El colágeno XV está presente en varios tejidos incluyendo la MB vascular y de las fibras musculares [182,183], participa en la organización de la microvasculatura [184] y mutaciones genéticas se han asociado con alteraciones en la morfología de los capilares, extravasación de eritrocitos y degeneración de músculo esquelético y cardíaco [156,184,185]. Por lo tanto, el papel de la degradación de estos colágenos en la desestabilización inicial de la MB inducido por las MPVSs es un aspecto que debe estudiarse más a fondo, al igual que las implicaciones que puede tener la degradación de este colágeno en el daño y reparación del tejido muscular en los envenenamientos.

Por otro lado, el colágeno XIV fue detectado en mayor cantidad en el exudado inducido por la BaP1, también fue detectado en todos los tiempos sin cambios significativos en el exudado inducido por el veneno completo, mientras que el colágeno XII apareció solamente a las 6 horas. Los colágenos XII y XIV son colágenos FACITS que conectan y estabilizan los colágenos fibrilares I y II con la MEC y la MB [181,186,187]; por ejemplo, en el músculo esquelético estos colágenos conectan la MB de las células musculares con el epimisio y el perimisio [188,189]. Existen estudios *in vitro* que han demostrado que los dominios adicionales DC de las MPVSs median la unión con los dominios A del vWF de los colágenos tipo XII y XIV [40]. Por otro lado, un estudio previo de proteómica que comparó dos MPVS tipo PI, una hemorrágica y otra no hemorrágica, no encontró diferencias en la cantidad de productos de degradación de estos dos colágenos presentes en el exudado
[109], lo que sugiere que la degradación de estos componentes no participa en el mecanismo hemorrágico de las MPVSs. Sin embargo, dado su función en la organización y estabilidad de los colágenos fibrilares, especialmente en las fibras musculares, el papel de la degradación de estos colágenos es un aspecto que debe ser considerado en los procesos de daño y reparación tisular que ocurre en el accidente ofidico.

Colágenos fibrilares

Con respecto a los colágenos fibrilares, tanto el colágeno I como el colágeno III se encontraron en los exudados inducidos por las tres MPVSs. Además, ambos colágenos, principalmente el colágeno I, aparecen desde tiempos tempanos en el exudado inducido por el veneno completo y su cantidad aumenta en tiempos tardíos. El colágeno I es un colágeno que forma fibras y se distribuye en tejido conectivo no cartilaginoso como piel y tejido conectivo muscular [181]. El aumento de los colágenos fibrilares en el exudado a las 24 h puede ser debido a la acción de las MMPs endógenas sintetizadas durante el curso del proceso inflamatorio en el tejido. En un estudio de proteómica que comparó una MPVS hemorrágica y una PLA2 miotóxica, se encontró una mayor cantidad de colágenos I y III en el exudado inducido por la PLA2 [108]. Las PLA2 miotóxicas inducen un rápido daño muscular [190] y una respuesta inflamatoria caracterizada por síntesis de citoquinas y MMPs e infiltrado celular [49,190,191], lo cual puede provocar la hidrólisis de los colágenos fibrilares por parte de las MMPs y otras proteasas derivadas de las células inflamatorias. Productos de degradación de colágenos fibrilares se han detectado en exudados inducidos por MPVSs [108,109,113]. En uno de estos estudios no se observaron diferencias luego de la administración de dos MPVSs tipo PI, una hemorrágica y otra no hemorrágica, las cuales son capaces de inducir una respuesta inflamatoria en el tejido [109].

Otras proteínas de MEC

En los exudados producidos tanto por las MPVSs como por el veneno completo, aparecen varias proteínas de la MEC, las cuales poseen diferentes funciones en la interacción célula-matriz y matriz-matriz. Una de estas proteínas es la fibronectina, la cual fue detectada en los exudados inducidos tanto por las MPVSs hemorrágicas, como por el veneno completo, sin diferencias importantes entre las tres MPVSs, ni en el tiempo. Estudios *in vitro* han demostrado la unión de MPVSs de tipo PIII con fibronectina [37]. La fibronectina puede encontrarse como fibronectina plasmática, la cual es producida por el hígado, y fibronectina tisular, la cual es producida por células tisulares e incorporada a la MEC. La fibronectina tisular interactúa con las células a través de la integrina $\alpha_5\beta_2$ y con otros componentes de la MEC y está relacionada con señales extracelulares que regulan la morfogénesis y diferenciación celular [167,192]. La presencia de fibronectina en el exudado puede ser consecuencia de la extravasación del plasma o puede ser debido a la proteólisis de la fibronectina presente en la MEC. De acuerdo con el análisis de inmunoquímica, la fibronectina aparece degradada en tiempos tempranos, lo cual puede ser debido a la acción de las MPVSs sobre la variante tisular de esta proteína; mientras que la fibronectina aparece completa en tiempos tardíos, lo cual puede deberse a la extravasación del plasma.

Otras proteínas que aparecen en el exudado inflamatorio inducido por las MPVSs y el veneno completo son la trombospondina-1 y -4, tenasina X, lumican, vitronectina y fibrilina-1 y -2, dermatopontin y proteoglycan 4. La mayoría de estos componentes están presentes en mayor cantidad en el exudado inducido por la MPVSs tipo PI; por otro lado, la mayoría sale en el exudado inducido por el veneno sin diferencias en el tiempo, a excepción de las fibrilinas que se detectaron en mayor cantidad a las 6 h. La trombospondina es una glicoproteína de la MEC con propiedades adhesivas; además, la tromospondina-1 participa en los procesos de hemostasia, inflamación, regeneración de tejidos y angiogénesis [193-196]. La tenasina X posee propiedades antiadhesivas y participa en el remodelamiento de la MEC durante los procesos de cicatrización y regeneración de los tejidos [197-199]. El lumican es un proteoglicano rico en leucina que participa en la unión de los colágenos fibrilares [156,200,201]. La vitronectina se une a la integrina $\alpha_{v}\beta_{3}$, promueve la adhesión y migración cellular y está implicada en la hemostasia [202-204]. Las fibrilinas son componentes importantes en la formación de las fibras elásticas del tejido conectivo [205-207]. Por lo tanto, la proteólisis de todos estos componentes por parte de las MPVSs o su liberación debido a alteraciones de la MEC puede tener consecuencias fisiológicas y

5. CONCLUSIONES

- Las MPVSs hemorrágicas de tipo PII y PIII se unen a componentes de la membrana basal vascular y colocalizan con el colágeno IV en capilares, VPC y arteriolas; por el contrario, la MPVS de tipo PI se distribuyó de manera difusa en el tejido.
- La Basparina, una MPVS no hemorrágica de tipo PIII, no se une a componentes de la membrana basal vascular.
- Las MPVSs hemorrágicas son capaces de degradar el colágeno tipo IV de la MB vascular, tanto en presencia como en ausencia de flujo sanguíneo, siendo más susceptible la MB de capilares en comparación con la de arteriolas y VPC.
- 4. La distribución más difusa de la BaP1, una MPVS hemorrágica de tipo PI, puede estar asociada con los efectos adicionales sobre otros componentes de la microvasculatura, como relocalización de la VE cadherina en VPC con aumento de la permeabilidad vascular, y aumento del tamaño de las hendiduras entre las células de músculo liso y pericitos en arteriolas y VPC.
- 5. El patrón similar de degradación del colágeno IV por parte de las tres MPVSs, la presencia de perlecan en cantidades similares en el exudado inducido por las tres MPVSs, y la dinámica de aparición del colágeno IV y perlecan en los exudados inducidos por el veneno completo, sugieren que el colágeno IV, y posiblemente el perlecan, puede ser blancos claves de las MPVSs en el mecanismo de acción hemorrágico de estas toxinas.
- 6. Los exositios presentes en las MPVSs tipo PII y PIII contribuyen a localizar estas enzimas cerca de proteínas específicas de la MEC, con lo cual son capaces de proteolizar una cantidad reducida de componentes de la MEC. Por el contrario, las MPVS tipo PI, al carecer de tales exositios, se difunden más libremente en los tejidos actuando de manera generalizada sobre una mayor cantidad de componentes de la MEC.
- La degradación y aparición de las proteínas de la MEC en el exudado inflamatorio inducido por el veneno de *B. asper* sigue un comportamiento dual: por un lado las proteínas de la MB (colágeno IV, perlecan y nidogén-2), y los colágenos no fibrilares

VI, XV y XVIII aparecen en estadíos tempranos del envenenamiento, debido a la rápida acción de las MPVSs presentes en el veneno, lo cual refleja el rápido daño a la microvasculatura y estructuras de la MEC. Por otro lado, los colágenos fibrilares aumentan en estadíos tardíos del envenenamiento, debido probablemente a la acción de las MMPs y posiblemente otras proteinasas endógenas como parte de la respuesta inflamatoria y los procesos de reparación de los tejidos.

6. RECOMENDACIONES

- Identificar los componentes de la membrana basal con los que interaccionan las MPVSs in vivo por medio de estudios de inmunomicroscopía electrónica.
- Determinar las secuencias en los exositios de las MPVSs que median la unión con componentes de la membrana basal.
- Estudiar los efectos patológicos que puede tener la unión de las MPVSs a la MB de las vénulas post-capilares y arteriolas en la microvasculatura.
- Estudiar los mecanismos involucrados y las consecuencias en la patogénesis del daño tisular de los efectos de las MPVSs sobre la relocalización de la VE cadherina y el efecto sobre las células de músculo liso vascular y pericitos.
- Utilizar otros protocolos de extracción u otros anticuerpos contra el perlecan para poder estudiar la degradación de esta proteína en homogenizados de tejidos y exudado inflamatorio por medio de estudios de inmunoquímica.
- Estudiar el papel de la degradación del colágeno VI y XV por parte de las MPVSs en los mecanismos de hemorragia y daño muscular que ocurren durante el envenenamiento.
- Estudiar el papel que juega la proteólisis de diferentes componentes de la MEC por parte de las MPVSs o su liberación como consecuencia del daño tisular, en el remodelamiento de la MEC y en los procesos de inflamación y de reparación de los tejidos.

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APÉNDICE

ARTÍCULOS CIENTÍFICOS PUBLICADOS

ARTÍCULO I



G OPEN ACCESS

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Tissue Localization and Extracellular Matrix Degradation by PI, PII and PIII Snake Venom Metalloproteinases: Clues on the Mechanisms of Venom-Induced Hemorrhage

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Abstract

Snake venom hemorrhagic metalloproteinases (SVMPs) of the PI, PII and PIII classes were compared in terms of tissue localization and their ability to hydrolyze basement membrane components in vivo, as well as by a proteomics analysis of exudates collected in tissue injected with these enzymes. Immunohistochemical analyses of co-localization of these SVMPs with type IV collagen revealed that PII and PIII enzymes co-localized with type IV collagen in capillaries, arterioles and post-capillary venules to a higher extent than PI SVMP, which showed a more widespread distribution in the tissue. The patterns of hydrolysis by these three SVMPs of laminin, type VI collagen and nidogen in vivo greatly differ, whereas the three enzymes showed a similar pattern of degradation of type IV collagen, supporting the concept that hydrolysis of this component is critical for the destabilization of microvessel structure leading to hemorrhage. Proteomic analysis of wound exudate revealed similarities and differences between the action of the three SVMPs. Higher extent of proteolysis was observed for the PI enzyme regarding several extracellular matrix components and fibrinogen, whereas exudates from mice injected with PII and PIII SVMPs had higher amounts of some intracellular proteins. Our results provide novel clues for understanding the mechanisms by which SVMPs induce damage to the microvasculature and generate hemorrhage.

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Author Summary

Local and systemic hemorrhage are typical manifestations of envenomings by viperid snakes. Hemorrhagic activity is due to the action of snake venom metalloproteinases (SVMPs) in the microvasculature, especially in capillaries. There are large differences in the hemorrhagic potential of SVMPs, depending on their domain composition. The present study compared PI, PII and PIII hemorrhagic SVMPs for their tissue distribution and their ability to cleave proteins of the extracellular matrix (ECM), especially those of the basement membrane (BM) that provides mechanical stability to microvessels. Observations indicate that PII and PIII SVMPs, which exert a high hemorrhagic activity, are preferentially located in microvessels, whereas PI SVMP is distributed in a more widespread fashion in the tissue. In addition, when these toxins are injected at doses that induce a similar hemorrhagic effect, they cleave type IV collagen to a similar extent, showing differences in the cleavage patterns of other ECM components, such as laminin, nidogen and type VI collagen. The analysis of the exudates resultant from the action of these SVMPs in the tissue revealed many similarities and some differences in the exudate proteomes. Overall our results indicate that hydrolysis of type IV collagen is a key event in the onset of microvessel damage, and that the ability of SVMPs to bind to microvessels greatly determines their hemorrhagic potential.

Introduction

Zinc-dependent enzymes of the M12 reprolysin family of metalloproteinases are abundant components in the venoms of snakes, especially from species classified in the family Viperidae [1]. Snake venom metalloproteinases (SVMPs) have undergone a complex process of molecular evolution after the recruitment in the venom gland of an ADAM-like enzyme, an event that occurred before the diversification of the advanced families of the superfamily Colubroidea [2–4]. Further events included gene duplication, domain loss and neofunctionalization through mutations in regions coding for surface-exposed residues [5]. Such complex evolutionary land-scape has generated a great diversity of SVMPs in snake venoms with a wide spectrum of biological activities. In addition, post-transcriptional and post-translational events further contribute to determine the final pattern of SVMPs in a particular venom [6].

On the basis of domain constitution, three main classes of SVMPs occur in viperid venoms [1]: class PI is comprised by enzymes containing only the metalloproteinase domain in the mature protein, including the canonical zinc-binding motif HEXXHXXGXXH followed by a Metturn motif. SVMPs of the class PII present a disintegrin domain following the metalloproteinase domain; in many enzymes, this disintegrin domain is proteolytically released from its precursor [1,7]. Class PIII SVMPs comprise, in addition to the metalloproteinase domain, a disintegrin-like (Dis-like) domain followed by a cysteine-rich domain (Cys-rich). Post-translational processing of precursors of some PIII metalloproteinases results in the release of the Dislike and Cys-rich domains (DC fragment) [1]. Further heterogeneity arises from the fact that some PII and PIII SVMPs occur as dimers, and some PIII enzymes are comprised of an additional subunit constituted by a C-type lectin-like protein, linked to the main proteinase chain by disulfide bonds [1].

These variations in domain composition have implications for the function of these enzymes and for their toxic profile. The non-metalloproteinase domains in PII and PIII SVMPs contain exosites that determine the binding of these enzymes to particular targets in the extracellular matrix (ECM), especially in microvessels, or in the plasma membrane of cells [8–14]. Immunohistochemical observations revealed a distinct pattern of distribution of PI and PIII SVMPs in the tissue [8]. In addition, the presence of these domains may prevent the inhibition of these SVMPs by the plasma inhibitor α2-macroglobulin [15,16], thus allowing them to act systemically after gaining access to the bloodstream. A potential consequence of the presence of these non-metalloproteinase domains is that PIII SVMPs generally have a greater hemorrhagic potency than PI SVMPs [17,18]. Moreover, although few PII SVMPs have been characterized in terms of hemorrhagic activity, two of them have been demonstrated as highly active hemorrhagic toxins [16,19]. Despite experimental evidence of differential binding of PI and PIII SVMPs to tissue structures *in vivo* and ECM proteins *in vitro*, a detailed comparative analysis of location of PI, PII and PIII hemorrhagic SVMPs in tissue is lacking.

The ability of hemorrhagic SVMPs to degrade basement membrane (BM) components has been known for many years, and it has been hypothesized that hydrolysis of BM proteins is a key event in the onset of microvascular damage and hemorrhage by SVMPs [20–23]. When comparing the patterns of hydrolysis of BM components *in vivo* and *in vitro* between hemorrhagic and non-hemorrhagic PI SVMPs from *Bothrops* sp venoms, a striking difference was found regarding degradation of type IV collagen, as this BM component was hydrolyzed by the hemorrhagic toxin but not by the non-hemorrhagic SVMP [22]. Since type IV collagen plays a key role in the mechanical stability of BM and hence of the capillary vessel structure [24–27], this observation is likely to have relevant functional implications regarding the mechanism of action of hemorrhagic SVMPs. It is therefore necessary to expand these studies to SVMPs of the classes PII and PIII to assess whether the pattern of hydrolysis of ECM components, particularly those of the BM, is similar to the one described for PI SVMPs or whether enzymes of different classes present different degradation patterns.

The combination of complementary analytical experimental tools is necessary to gain a deeper understanding on the mechanism of action of hemorrhagic SVMPs. In the present study we explored the patterns of tissue localization of PI, PII and PIII SVMPs using an ex vivo model in the cremaster muscle of mice and immunofluorescence confocal microscopy. In parallel, the patterns of BM protein degradation on skin and muscle in vivo were investigated by immunochemical analysis of tissue homogenates and exudates. Finally, a proteomic analysis of exudate collected from the tissue affected by the SVMPs was performed. Such proteomic analysis constitutes a 'window' through which details of toxin-induced tissue alterations, unobserved by more traditional histological analyses, can be detected [28,29]. Our findings reveal a distinct pattern of tissue localization of these SVMPs, with PII and PIII enzymes showing a close association with the microvasculature, in contrast to PI SVMP, which had a more widespread distribution in the tissue. Furthermore, variable patterns of degradation between the SVMPs were observed for nidogen and laminin, whereas type IV collagen was hydrolyzed to a similar extent by the three enzymes. In addition, proteomic analysis of exudate showed variations which suggest differences in the pathological effects induced by these toxins. In summary, these observations provide for a new and more complete understanding the mechanism of microvessel damage and hemorrhage induced by SVMPs.

Methods

Isolation of SVMPs and characterization of a new PIII from the venom of C. simus

The PI SVMP BaP1 was isolated from the venom of *Bothrops asper* as described by Gutiérrez et al. [30] and Watanabe et al. [31] by a combination of ion-exchange chromatography on CM-Sepharose, followed by affinity chromatography on Affi-gel Blue. The dimeric PH SVMP BlatH1 was purified from the venom of *Bothriechis lateralis* as described by Camacho et al.

[16] by ion-exchange chromatography on DEAE-Sepharose, followed by hydrophobic interaction chromatography on Phenyl Sepharose and gel filtration on Superdex 200 10/300GL. A novel PIII SVMP was purified from the venom of adult specimens of the Central American rattlesnake *Crotalus simus*. The venom was fractionated by ion-exchange chromatography on a DEAE-Sepharose column using a BioLogic LP chromatography system (Bio-Rad). After washing the column with initial buffer (0.25 mM Tris-HCl, 2.5 mM CaCl₂, pH 7.0), a linear gradient was developed from 0 to 0.35 M NaCl in the starting buffer. The last fraction eluted, consisting mainly of a 55 kDa hemorrhagic toxin, was further purified by gel filtration chromatography on a SuperdexTM 200 10/300GL (GE Healthcare, LifeSciences) column (10 x 300 mm) previously equilibrated with 0.05 M Tris-HCl, 5 mM CaCl₂, pH 5.8, buffer using an ÄKTA FPLC (GE Healthcare, LifeSciences).

Homogeneity and molecular mass of the *C. simus* SVMP were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), run under reducing and non-reducing conditions [32]. SDS-PAGE electrophoresis was performed on 15% Tris-HCl polyacrylamide gel and staining was performed, either with Coomassie Brilliant Blue for total protein, or with Pro-Q Emerald 300 Glycoprotein Stain Kit (Molecular Prohes) for detection of carhohydrates. Proteolytic activity was assessed on azocasein (Sigma) as described by Wang et al. [33]. Hemorrhagic activity was evaluated by injecting various amounts of the SVMP intradermally in mice and measuring, after 2 h, the diameter of the hemorrhagic lesion in the internal side of the skin [34]. The Minimum Hemorrhagic Dose (MHD) corresponds to the amount of enzyme that induces a hemorrhagic spot of 10 mm diameter 2 h after injection. In addition, the ability of this enzyme to induce pulmonary hemorrhage was assessed by injecting 50 µg of the enzyme by the intravenous route in mice. One hour after injection, mice were sacrificed by an overdose of xylazine and ketamine, and lungs were dissected out and routinely processed for embedding in paraffin and staining with hematoxylin-eosin.

For tryptic peptide mapping and internal peptide sequence determination, purified PIII-SVMP was excised from a Coomassie Brilliant Blue-stained SDS-PAGE and subjected to automated reduction (10 mM dithiothreitol) and alkylation (50 mM iodacetamide), followed by overnight trypsin digestion (66 ng/µL of sequencing-grade porcine trypsin (Promega) in 25 mM ammonium bicarbonate, 10% acetonitrile; 0.25 µg/sample) in a ProGestTM Protein Digestion Workstation (Genomics Solutions) following manufacturer's instructions. For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization (ESI) mass spectrometric analysis using a QTrapTM 2000 mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q₀ trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for collision-induced dissociation MS/MS experiments were as follows: Q1- unit resolution; Q1-to-Q2 collision energy—30–40 eV; Q3 entry barrier—8 V; LIT (linear ion trap) Q3 fill time—250 ms; and Q3 scan rate—1000 amu/s. Product ion spectra were interpreted manually.

The complete amino acid sequence of *C. simus* PIII SVMP was determined by a combination of tryptic peptide MS/MS sequencing and cDNA cloning from a venom gland cDNA library previously constructed and used for profiling the venom gland transcriptomes of Costa Rican snakes by 454 pyrosequencing [35]. The amplification mixture contained, in a final volume of 50 µL: 1 µL of cDNA library; 1 µL of a 10 µM stock solution of each, forward (Fw: 5-AAC CCC TTC AGA 'TTC GTT GAG-3') and reverse (Rv: 3'-ATA GGC TGT AGC CAC ATC AAC-5') primers derived from the amino acid sequences NPFRFVE and VDVATAY, respectively, determined by *de novo* MS/MS tryptic ion sequencing, these amino acid sequences are highly conserved in the N- and C-termini of PIII-SVMPs, respectively; 0.25 µL of DNA
polymerase (GoTaq, Promega); 1 µL of 10 mM dNTPs; 2 µL of 25 mM MgCl₂; 5 µL of 5x buffer; and 13.75 µL of DNAse-free deionized water. The PCR protocol included initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation (10 s at 94°C), annealing (45 s at 55°C) and extension (120 s at 72°C), and a final extension for 7 min at 72°C. The amplified fragment was purified from an agarose gel using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and cloned in a pCR-XL-TOPO vector (Invitrogen). *E. coli* DH5 cells (Novagen, Madison, WI, USA) were transformed by electroporation using an Eppendorf 2510 electroporator following the manufacturer's instructions. Positive clones, selected by growing the transformed cells in Luria-Broth (LB) medium containing 10 µg/ml ampicillin, were confirmed by PCR amplification using the above primers, and the PCR-amplified fragments were sequenced using an Applied Biosystems model 377 DNA sequencer.

Ethics statement

All *in vivo* experiments were performed in CD-1 mice. The experimental protocols involving the use of animals in this study were approved by the CICUA (University of Costa Rica) and meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS).

Experiments ex vivo

Immunolocalization of SVMPs in muscle tissue. The mouse cremaster muscle was used to study the distribution and immunolocalization of the SVMPs because it is possible to obtain high resolution images of longitudinal blood vessels by confocal microscopy due to the transparency and thinness of this tissue. Groups of three male mice were killed by cervical dislocation and the cremaster muscle was dissected out. The isolated muscles were incubated in PBS for 15 min with either BaP1 (PI, 30 µg), BlatH1 (PII, 3.5 µg) or CsH1 (PIII, 15 µg) SVMPs labeled with Alexa Fluor 647 according to the Microscale Protein Labeling Kit (Molecular Probes A30009). These doses were selected as to induce a hemorrhage in the cremaster muscle of similar intensity to that described previously by intravital inicroscopy for the PI SVMP BaP1 [36]. Control tissues were incubated with unlabeled toxins. After incubation, tissues were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. Fixed whole tissues were incubated for 4 h at room temperature in blocking and permeabilization solution (12.5% goat serum, 12.5% fetal bovine serum and 0.5% Triton X-100 in PBS). Then, the tissues were immunostained overnight at 4°C with rabbit anti-collagen type IV polyclonal antibody at a dilution of 1:100 (Abcam ab19808), and Cy3-labeled mouse anti-actin a smooth muscle monoclonal antibody at a dilution of 1:200 (clon 1A4, Sigma C6198) to visualize the vascular basement membrane and smooth muscle/pericytes, respectively. After three washes in PBS, tissues were incubated for 4 h at 4°C with goat anti-rabbit polyclonal antibody labeled with Alexa Fluor 488 at a dilution of 1:200 (Invitrogen A11034). In order to ascertain whether labeling of SVMPs had an effect on their enzymatic activity, proteolytic activity of the labeled enzymes was quantified on gelatin with the EnzChek Gelatinase Assay Kit (Molecular Probes E-12055) at different times during 24 h.

Immunostained tissues were mounted on glass slides and visualized using a Zeiss LSM 5 Pascal laser-scanning confocal microscope (Carl Zeiss Ltd) incorporating a 10X objective (numerical aperture 0.3) and 63X oil objective (numerical aperture 1.4). At least four images of post-capillary venules (PCV), arterioles and capillaries per tissue were taken in 3 dimensions at a resolution of 1,024 × 1,024 dpi corresponding to a voxel size of 0.14 × 0.14 × 0.38 μ m in the X × Y × Z plans, respectively using the 63X objective. Three-dimensional reconstitution of the images and analysis of co-localization of the SVMPs with collagen IV were carried out using IMARIS x64 7.4.2 image analysis software, which employs the approach developed by Costes et al. [37]. A region of interest (ROI) in the pixel intensity of 10 was defined to calculate the threshold for each label with the automatic function of the software. The program IMARIS analyzes the intensity of each label by voxels defined as a prism with the pixel in the base and the thickness of the confocal section in the height. The results of co-localization were expressed as the percentage of material co-localized which takes into account the number of voxels co-localized and the Pearson's correlation coefficient, which reflects the correlation between intensities in the co-localized voxels. The Pearson's coefficient varies between +1 and -1, where values near 1 indicate a direct correlation, and values near 0 indicate no correlation. The percentage of co-localization may overestimate the extent of co-localization, while the Pearson's coefficient may underestimate the extent of co-localization since it takes into account that the intensity of the two labels varies together. Hence, it is important to analyze both values in co-localization studies.

Experiments in vivo

Immunochemical detection of ECM proteins in the skin and exudate. Groups of five mice were injected intradermally in the ventral abdominal region with either BaP1 (PI, 75 μ g), BlatH1 (PII, 1.5 μ g) or CsH1 (PIII, 35 μ g) SVMPs, dissolved in 100 μ L of PBS. These doses were selected as to induce a similar hemorrhagic area in the injected skin, since these enzymes have highly different hemorrhagic activity, i.e. Minimum Hemorrhagic Doses [16,30], [this work]. The control group received 100 μ L of PBS alone. After 15 min, mice were sacrificed by CO₂ inhalation, their skin was removed, and an area of 12 mm diameter in the site of the injection was dissected out. In order to prepare a pool, all skin fragments from each treatment were combined the placed in liquid nitrogen and pulverized until fine particles were obtained. Each pool was resuspended in 1.5 mL of extraction buffer (25 mM Tris-HCl, 150 mM NaCl, 8 M urea, 40 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 7.4) with a tablet of protease inhibitor cocktail (Roche) per 10 mL of buffer. One hour after incubation under stirring at 4°C, samples were centrifuged at 5,200 g for 5 min and the supernatant was diluted 1:2 with water and stored at -70°C until Western blot analysis was performed.

In another set of experiments, groups of five mice were injected in the right gastrocnemius with either BaP1 (PI, 75 μ g), BlatH1 (PII, 3 μ g) or CsH1 (PIII, 50 μ g) SVMPs, dissolved in 50 μ L of PBS. These doses were selected as they induce a similar extent of hemorrhagic activity in muscle. After 15 min of injection, mice were sacrificed by CO₂ inhalation, and a 5 mm incision was made with a scalpel in the skin overlying the injected muscle. Immediately, the sectioned skin was opened and a heparinized capillary tube was introduced under the skin to collect the wound fluid [28]. An approximate volume of 20–50 μ L of exudate was collected from each mouse. Exudate samples were then pooled and lyophilized.

For immunoblotting, 10–20 µL of each skin homogenate sample, or 100 µg protein of each exudate sample, were separated under reducing conditions on 4–15% Tris–HCl SDS-PAGE gradient gels, and transferred to nitrocellulose membranes. Immunodetection was performed by incubating the membranes overnight at 4°C under stirring with either rabbit anti-collagen type VI polyclonal antibody at a dilution of 1:2,000 (Millipore AB7821), rabbit anti-collagen type IV polyclonal antibody at a dilution of 1:1,000 (Abcam ah6586), rabbit anti-laminin polyclonal antibody at a dilution of 1:1,000 (Abcam ab11575), or rabbit anti-nidogen 1 polyclonal antibody at a dilution of 1:1,000 (Abcam ab14511). The anti-GAPDH antibody at a dilution of 1:1,000 (Abcam ab9485) was used as loading control for immunoblotting of the skin homogenates samples. The reaction was detected using an anti-rabbit peroxidase antibody at a dilution of 1:10,000 (Jackson ImmunoResearch) and the chemiluminescent substrate Lumi-Ligth

(Roche). The images were obtained with the ChemiDoc XRS+ System (BioRad) and the analysis was performed with the ImageLab software (BioRad).

Analysis of the proteomics of exudates

Lyophilized wound exudate samples were re-suspended in water and protein quantification was performed using micro BCA protein assay kit (Thermo Scientific) Twenty micrograms of protein were then resuspended in Laemmli buffer, applied to a 12% precast electrophoresis gel (Bio-Rad), separated, and stained with Coomassie Brilliant Blue. Gel lanes were cut in ten equal size slices. Gel slices were destained for 3 h and the proteins reduced (10 mM DTT) and alkylated (50 mM iodoacetamide) at room temperature. Gel slices were washed with 100 mM ammonium bicarbonate, deliydrated with acetonitrile and dried in a speed vac. Hydration of the slices was performed with a solution of Promega modified trypsin (20 ng/µL) in 50 mM ammonium bicarbonate for 30 min on ice. Excess trypsin solution was removed and the digestion was carried on for an additional 18 h at 37°C. Tryptic peptides were twice extracted from gel slices with 30 µL of a 50% acetonitrile/5% formic acid solution. The combined extracts were dried to a volume of 15 µL for mass spectrometric analysis. LC/MS/MS was performed using a Thermo Electron Orbitrap Velos ETD mass spectrometer system. Analytical columns were fabricated in-house by packing 0.5 cm of irregular C18 Beads (YMC Gel ODS-A, 12 nm, I-I0-25 um) followed by 7.5 cm Jupiter 10 µm C18 packing material (Phenomenex, Torrance, CA) into 360 x 75 µm fused silica (Polymicro Technologies, Phoenix, AZ) behind a bottleneck. Samples were loaded directly onto these columns for the C18 analytical runs. Aliquots of 7 µL were loaded onto the column for each analysis and eluted into the mass spectrometer at 0.5 µL/min using an acetonitrile/0.1 M acetic acid gradient (2-90% acetonitrile over 1 h). The instrument was set to Full MS (m/z 300-1600) resolution of 60,000 and programmed to acquire a cycle of one mass spectrum followed by collision-induced dissociation (CID) MS/MS performed in the ion trap on the twenty most abundant ions in a data-dependent mode. Dynamic exclusion was enabled with an exclusion list of 400 masses, duration of 60 seconds, and repeat count of 1. The electrospray voltage was set to 2.4 kV, and the capillary temperature was 265°C.

Peak lists were generated from the raw data using the Sequest search algorithm in Proteome Discoverer 1.4.1 against the Uniprot Mouse database from July 2014. Spectra generated were searched using carbamidomethylation on cysteine as a fixed modification, oxidation of methionine as a variable modification, 10 ppm parent tolerance and 1 Da fragment tolerance. All hits were required to be fully tryptic. The results from the searches were exported to Scaffold (version 4.3.2, Proteome Software Inc., Portland, OR). Scaffold was used to validate MS/MS based peptide and protein identifications and to visualize multiple datasets in a comprehensive manner. Protein identifications were filtered using Xcorr cutoff values dependent on charge state (+1>1.8, +2>2.2, +3>2.5 and +4>3.5). Confidence of protein identification in Scaffold is displayed as a Probability Legend with green coloration indicative of over 95% confidence and yellow as 80% to 94% confidence. Relative quantization of proteins was accomplished by summing all data from the 10 gel slices for a particular sample in Scaffold and then displaying the Quantitative Value from the program. This number gives an average total of non-grouped spectral counts for a protein divided hy the total non-grouping spectral counts for the 10 mass spectral runs from the gels slices from each lane (http://www.proteomesoftware.com/). This format of presentation allows for a relative quantitative comparison between a specific protein from different samples and to a certain degree gives some measure of relative abundance between proteins generated from the mass spectrometric analysis of the 10 gel slices for a particular exudate sample. Portions of the data were further analyzed manually to determine if mass

spectra were derived from proteins migrating in the gel at their expected molecular mass or at a lower mass.

Results

Characterization of a new PIII SVMP from the venom of C. simus

Through a combination of ion-exchange chromatography on DEAE-Sephadex and gel filtration, a novel hemorrhagic SVMP was purified to homogeneity from the venom of adult specimens of the Central American rattlesnake *C. simus* (Fig.1A and 1B). This SVMP is hereby named CsH1. It is a monomeric glycosylated protein with a molecular mass of 55 kDa (Fig.1A and 1B). The cDNA-deduced amino acid sequence was identified by BLAST analysis as Gen-Bank accession number DQ164403 (the species *Crotalus simus* was previously named *Crotalus durissus durissus*), and various tryptic peptide ion sequences gathered by *de novo* MS/MS sequencing of an in-gel digested Coomassie Brilliant Blue-stained SDS-PAGE band of the purified PIII SVMP (Table 1) confirmed the assignment. The enzyme possesses a zinc



Fig 1. (A), (B) SDS-PAGE of *Crotalus simus* PIII SVMP and crude venom. Samples were run on a 12% gel, and stained with (A) Coomassie blue or (B) Pro-Q Emerald 300 glycoprotein stain (Molecular probes). Lane 1: SVMP, reducing conditions; Iane 2: SVMP, non-reducing conditions; Iane 3: *Crotalus simus* venom, reducing conditions; Iane 4: *Crotalus simus* venom, non-reducing conditions. The P-III metalloproteinase is a major component of the venom; it is glycosylated and has a molecular mass of 55 kDa. (C) and (D) Light micrographs of sections of lung tissue from mice injected intravenously with either saline solution (C) or 100 µg of C. *simus* PIII SVMP. Mice were sacrificed one h after injection and tissue samples were obtained and routinely processed for embedding in paraffin and further staining with hematoxylin-eosin. Notice prominent hemorrhage in the pulmonary tissue in (D) (arrow). 125 X.

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m/z	z	Amino acid sequence
275.3	2+	CADGK
291.8	2+	JYCK
354.7	2+	SGTECR
359.8	2+	TDJJTR
401.3	2+	GMVJPGTK
417.3	2+	NNDDJDK
467.8	2+	ZKYNPFR
526.3	2+	GNYYGYCR
615.8	2+	DNSPGQNNPCK
649.9	2+	MFYSNEDEHK
760.8	3+	YMYJHVAJVGJEJWSNEDK
766.4	2+	VJGJAYVGSMCHPK
801.3	2+	MYEJANTVNDJYR
776.2	2+	VCSNGHCVDVATAY
885.4	2+	SGSQCGHGDCCEQCK
684.6	3+	JTVKPEAGYTJNAFGEWR
869.8	3+	ZKYNPFRFVEJVJVVDKAMVTK
926.3	3+	ASMSECDPAEHCTGOSSECPADVFH

Table 1. Tryptic peptide ion sequences obtained by de novo CID-MS/MS sequencing of Crotalus simus PIII SVMP.

J, Isoleucine (I) or Leucine (L); Z, pyroglutamic acid (2-oxo-pyrrolidone carboxylic acid). Cysteine residues (G) are carbamidomethylated.

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metalloproteinase domain, a disintegrin-like domain and a cysteine-rich domain, hence corresponding to a PIII SVMP. The SVMP has proteolytic activity on azocasein and was hemorrhagic using the mouse skin assay, with a MHD of 2.2 µg. Both proteolytic and hemorrhagic activities were abrogated by incubation with EDTA. The enzyme was also able to induce hemorrhage in lungs after intravenous injection (Fig.1C and 1D); thus it induces both local and systemic hemorrhage.

Inmunolocalization of SVMPs in the tissue

When equi-hemorrhagic amounts of either PI, PII or PIII SVMPs were incubated for 15 min with the isolated cremaster muscles *ex vivo*, a clear difference in the distribution of the toxins was observed (Fig 2A). BlatH1 (PII) and CsH1 (PIII) SVMPs were preferentially localized in the basement membrane of blood vessels, as evidenced by co-localization with collagen IV. In contrast, localization of BaP1 (PI) SVMP was observed widespread in the tissue and to a lesser extent in the vascular basement membrane (Fig 2A). No fluorescence was detected in the control tissues incubated with unlabeled SVMPs. This localization of BlatH1 (PII) and CsH1 (PIII) SVMPs was noted in post-capillary venules (PCV) and also in arterioles and capillaries (Fig 2B). The proteolytic activity on gelatin of the three SVMPs after labeling with Alexa Fluor 647 was between 75–85% as compared to control unlabeled enzymes, thus indicating that labeled SVMPs remained functionally active.

The analysis of co-localization (Fig 2C and 2D) showed a higher percentage of co-localization (around 40%) and Pearson's coefficient (around 0.4) for BlatH1 (PII) and CsH1 (PIII) SVMPs with collagen IV of the vascular basement membrane in PCV, arterioles, and capillaries as compared with BaP1 (PI) SVMP (p<0.001). No significant differences were observed between co-localization of PII and PIII SVMPs with collagen IV in the basement membrane of PLOS NEGLECTED TROPICAL DISEASES

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Fig 2. Immunolocalization of SVMPs with vascular basement membrane on cremaster muscle ex vivo. Isolated cremaster muscles were incubated for 15 min with equi-hemorrhagic amounts of either BaP1 (PI, 30 µg), BlatH1 (PII, 3.5 µg) or CsH1 (PIII, 15 µg), SVMPs labeled with Alexa Fluor 647 (blue). Control tissues were incubated with the SVMPs without labeling and no fluorescence was detected. Whole tissues were fixed with Alexa Fluor 647 (blue). Control tissues were incubated with the SVMPs without labeling and no fluorescence was detected. Whole tissues were fixed with 4% paraformaldehyde and immunostained with anti-collagen IV following the secondary antibody labeled with Alexa Fluor 488 (green). Tissues were visualized in a Zeiss LSM 5 Pascal laser-scanning confocal microscope. Three-dimensional reconstitution of the images and analysis of co-localization were carried out with the IMARIS x64 7.4.2 software as described in Methods, (A) Distribution of the SVMPs in the cremaster muscle tissue. Scale bar represents 150 µm. (B) White areas represent co-localization of the SVMPs (blue) with collagen IV (green) of vascular basement membrane in PCV, arterioles, and capillaries. Scale bar represents 20 µm. Results are expressed as the mean ± SEM of (C) percentage of material of SMVPs co-localized with collagen IV of vascular basement membrane, and (D) Pearson's correlation coefficient of at least four vessels type per tissue (n = 3). *p<0.001 when compared with BaP1 (PI) SVMP for postcapillary venules (PCV), arterioles, and capillaries.

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arterioles, capillaries, and PCV. Moreover, no significant differences were observed between co-localization of each SVMPs in the basement membrane of arterioles, capillaries, and PCV.

Immunochemical analysis of BM-associated proteins in the skin and in exudates

Hemorrhagic lesions of similar extent and intensity were observed in the skin of mice 15 min after the injection of 75 μ g, 1.5 μ g and 35 μ g of the PI, PII and PIII SVMP, respectively, as expected when accounting for the highly different MHDs of these SVMPs. Homogenates of the sections of hemorrhagic skin were analyzed by immunoblot for the detection of several ECM components. With regard to the immunodetection of type IV collagen, samples from control skin injected with saline solution showed a predominant band of 107 kDa, with additional faint bands of 216 kDa, 176 kDa, 165 kDa, 117 kDa, and 97 kDa (Fig 3A). A conspicuous degradation band of 97 kDa was observed in samples from skins injected with the three SVMPs, together with a reduction in the intensity of the 107 kDa band (Fig 3A). The intensity of the 97 kDa band had the following order: PI > PII > PIII. Regarding type VI collagen, PI SVMP induced a



Fig 3. Western blot analysis of basement membrane components in skin homogenates. Groups of five mice were injected by intradermal route in the ventral abdominal region with either BaP1 (PI, 75 µg), BlatH1 (PII, 15 µg), CsH1 (PII, 35 µg) SVMPs or PBS (lane C). After 15 min, mice were sacrificed, their skin was removed, and an area of 12 mm diameter was dissected out. Tissues of the same group were homogenized and centrifuged, and the supernatant collected. Then, 10–20 µL of each skin homogenate sample were separated under reducing conditions on 4–15% Tris-HCI SDS-PAGE gradient gels, and transferred to nitrocellulose membranes. Immunodetection was performed with (A) anti-collagen type IV, (B) anti-collagen type VI, (C) anti-laminin, and (D) anti-nidogen 1. The anti-GAPDH antibody was used as loading control. The reaction was detected using an anti-rabbit peroxidase antibody and a chemiluminescent substrate. Images were obtained with the ChemiDoc XRS+ System (BioRad).

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reduction in the intensity of the predominant band of 216 kDa, whereas the PII and PIII SVMPs did not seem to hydrolyze this chain (Fig 3B). Degradation products of 160 kDa and 140 kDa were observed in samples from mice injected with PI SVMP, whereas the intensity of these bands in samples corresponding to the other two SVMPs was less pronounced (Fig 3B).

When laminin was immunodetected in skin homogenates, a predominant band of 167 kDa was observed, with additional bands of 270 kDa and 350 kDa (Fig 3C). The PI SVMP induced a greater hydrolysis of laminin, with the appearance of degradation products of 225 kDa and 50 kDa. The former band was not observed in samples from PII and PIII-treated mice (Fig 3C). The three SVMPs differ in their degradation of nidogen (Fig 3D). Samples from skin of nice injected with saline showed a predominant band of 135 kDa and few additional minor bands (Fig 3D). PI SVMP induced extensive degradation of nidogen, as evidenced by the disappearance of the 135 kDa band, and the appearance of a 47 kDa degradation band. A lower extent of hydrolysis was observed with the PII and PIII SVMPs. There was a reduction in the 135 kDa band, and the appearance of 100 kDa (in PIII SVMP) and of 47 kDa in samples injected with either enzyme (Fig 3D).

Western blot analysis of exudates collected 15 min after injection of the enzymes revealed both similarities and differences between the three SVMPs. A relatively similar pattern was observed in the case of type IV collagen, with the presence of a predominant band of 90 kDa (Fig 4A). Samples from mice injected with PI and PII SVMPs showed a band of 20 kDa, which was not present in the case of PIII SVMP. In contrast, PIII SVMP generated a band of 140 kDa not observed in the case of the other two enzymes. A highly variable pattern of immunoreactivity was observed in exudates when tested for type VI collagen degradation products (Fig 4B). PI SVMP generated fragments of 225 kDa, 150 kDa, and 100 kDa. On the other hand, bands of 220 kDa and 200 kDa were observed in samples from PII SVMP-injected mice, and bands of 225 kDa, 200 kDa, 160 kDa, 140 kDa, and 40 kDa were present in exudates as a consequence of



Fig 4. Western blot analysis of basement membrane components in exudates collected from the gastrocnemius. Groups of five mice were injected in the right gastrocnemius with either BaP1 (PI, 75 µg), BlatH1 (PII, 3 µg), or CsH1 (PIII, 50 µg) SVMPs. After 15 min, mice were sacrificed, a 5 mm incision was made in the skin overlying the injected muscle, and a heparinized capillary tube was introduced under the skin to collect the wound exudate fluid; exudate samples from a single treatment were then pooled. Afterwards, 100 µg of protein of each sample was separated under reducing conditions on 4–15% Tris– HCI SDS-PAGE, and transferred to nitrocellulose membranes. Immunodetection was performed with (A) anti-collagen type IV, (B) anti-collagen type VI, (C) anti-famimin, and (D) anti-nidogen 1. The reaction was detected using an anti-rabbit peroxidase antibody and a chemiluminescent substrate. Images were obtained with the ChemiDoc XRS+ System (BioRad).

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the action of PIII SVMP (Fig 4B). In the case of laminin, exudate from mice injected with PI SVMP showed bands of 275 kDa, 230 kDa, 200 kDa, 150 kDa, 105 kDa and 50 kDa (Fig 4C). On the other hand, PII and PIII SVMPs generated a similar pattern of immunoreactive bands of 275 kDa, 190 kDa, and 50 kDa, with an additional band of 140 kDa in the case of PIII SVMP (Fig.4C). Analysis of nidogen in exudates showed the presence of immunoreactive bands of 140 kDa and 50 kDa in the case of PII SVMP, and of 50 kDa in the case of PIII SVMP, whereas no immunoreactive bands were observed in the exudate of mice injected with PI SVMP (Fig.4D).

Proteomic characterization of exudates

A similar protein pattern was observed when exudates were separated by 1D SDS-PAGE. From the mass spectral analysis of the gel bands, a total of 297, 354 and 322 proteins, with protein identification probability greater than 95% and minimum of two peptides, were identified in exudates collected from mice injected with PI, PII and PIII SVMPs, respectively (S1 Table includes the complete report of all the proteins identified in the exudates). The most abundant proteins identified based on their quantitative value (see http://www.proteomesoftware.com/ for full description of term) were analyzed. Prnteins were classified within the following groups, and subgroups: (a) serum proteins; (b) proteins of the coagulation cascade; (c) proteinase inhibitors of plasma; (d) intracellular proteins; (e) keratins; (f) ECM proteins; and (g) membrane-associated proteins.

Serum proteins, including coagulation factors and proteinase inhibitors. S2–S1 Tables depict the quantitative values of serum proteins, proteins of the coagulation cascade and proteinase inhibitors, respectively. In general, similar values were observed for the vast majority of proteins in exudates collected from mice injected with the three SVMPs, especially in those with greatest quantitative values. Relatively minor differences were observed in the serum proteins (S2 Table). The highest variations occurred in fibrinogen, with higher amounts in the case of PI and PII SVMPs (S3 Table), and in some apolipoproteins, especially apolipoprotein B-100, of which the PI SVMP induced great amounts in the exudate (S2 Table). No relevant differences were noticed between SVMPs regarding the amounts of serum proteinase inhibitors (S4 Table).

Intracellular proteins. Similar amounts of hemoglobin chains occurred in exudates obtained from mice injected with the three SVMPs (S5 Table), as expected from the similar extent of hemorrhage induced. Likewise, similar amounts of many other proteins characterized the three types of exudates. However, there was a group of intracellular proteins in which higher values were detected in exudates from mice injected with PII and PIII SVMPs (S5 Table). Results on keratins are presented separately (S6 Table) owing to the relevance of skin damage induced by snake venoms. PII SVMP, and especially PI SVMP, induced a higher amount of keratins in exudates than PIII SVMP (S6 Table).

ECM proteins. As shown in Table 2, no differences were observed in detected BM proteins (heparin sulfate proteoglycan and nidogen). In contrast, there were differences in other ECM proteins. There was a tendency for higher values in several proteins in exudates collected from mice injected with the PI SVMP, such as tenascin, vitronectin, type VI collagen, type XIV collagen, type III collagen, and thrombospondin-4 (Tahle 2). Proteolysis of ECM proteins was analyzed on the basis of the range of molecular masses of the bands in SDS-PAGE gels. Identification of the various proteins in ranges of molecular mass lower than the known mass of the native proteins were considered degradation fragments, and the percentage of the total amount of each protein corresponding to hydrolyzed bands was estimated. As shown in Table 3, Results indicate that various types of collagens detected were degraded by the three SVMPs, with the exception of collagen type

Protein	Accession Number	Mol. Mass	Quantitative value		
			P-1	P-II	P-11
Fibronectin	P11276	273 kDa	87	34	43
Tenascin X	E9Q2T3	340 kDa	10	0	0
Basement membrane-specific heparan sulfate proteoglycan core protein	B1B0C7 (+1)	469 kDa	4	3	2
Lumican	P51885	38 kDa	8	7	7
Vitronectin	P29788	55 kDa	7	5	2
Collagen alpha-1 (I) chain	P11087	138 kDa	3	4	4
Protein Col6a3	E9PWQ3	354 kDa	8	1	2
Collagen alpha-1(XIV) chain	B7ZNH7 (+3)	193 kDa	9	3	2
Thrombospondin-4	Q9Z1T2	106 kDa	4	0	1
Nidogen-1	P10493	137 kDa	3	2	2
Collagen alpha-1(III) chain	P08121	139 kDa	5	1	з
Collagen alpha-1(XV) chain	A2AJY2 (+1)	138 kDa	0	0	3
Collagen alpha-2(I) chain	Q01149	130 kDa	3	0	3

Table 2. Extracellular matrix proteins identified in wound exudate collected from mice injected with PI, PII or PIII SVMPs.

Values in bold and underlined correspond to proteins for which at least one SVMP induced an increment of at least three times as compared to another SVMP.

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XIV, which was degraded 100% by the P-III, 33% by the PI and 0% by the PII. Regarding BM proteins, heparan sulfate proteoglycan and nidogen-1 were similarly degraded hy the three SVMPs. Lumican was not degraded by any SVMP. Fibronectin was degraded to a higher extent by PI and PIII than by PII. Thrombospondin 4 was degraded only by the PI SVMP.

Membrane-associated proteins. Seven membrane-associated proteins were detected in the exudates. With one exception, the amounts of these proteins did not differ more than 3-fold in exudates from mice injected with the three types of SVMPs (S7 Table).

T	able	3. 1	Degrada	tion o	of extracellular	matrix prote	ins identified	in wound e	exudates (s	see Methods	section fo	or details).

Proteins	Accession number	Mol. mass	Percentage degradation		
			PI	Pll	PIII
Fibronectin	P11276	273 kDa	45%	6%	76%
Tenascin X	E9Q2T3 (+1)	340 kDa	11%		*
BM-specific heparan sulfate proteoglycan core protein	B1B0C7 (+2)	469 kDa	50%	67%	50%
Vitronectin	P29788	55 kDa	100%	20%	50%
Lumican	P51885	38 kDa	0%	0%	0%
Thrombospondin-4	Q9Z1T2	106 kDa	75%		
Nidogen-1	P10493	137 kDa	100%	100%	100%
Collagen alpha-1(XV) chain Col15a1	A2AJY2 (+3)	138 kDa	100%	a	100%
Collagen alpha-1(I) chain Coi1a1	P11087	138 kDa	100%	100%	100%
Collagen alpha-1(III) chain Col3a1	P08121	139 kDa	100%	100%	100%
Collagen alpha-1(XIV) Col14a1	B7ZNH7 (+3)	193 kDa	33%	0%	100%
Collagen alpha-2(I) chain Col1a2	Q01149	130 kDa	100%	*	100%
Col6a3 (fragment)	D3YWD1 (+2)	186 kDa	100%	100%	•

* Not detected.

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Discussion

This study analyzed, from a comparative perspective, the tissue localization and the degradation of ECM proteins and other plasma and cellular proteins in the tissues of mice injected with hemorrhagic PI, PII and PIII SVMPs. Since one of the main goals of this work was to gain further insights into the mechanisms of SVMP-induced microvessel damage leading to hemorrhage, the doses of SVMPs injected were standardized as to induce the same extent of hemorrhagic lesions. It was hypothesized that, in these experimental conditions, the ECM proteins whose hydrolysis is directly responsible for microvessel damage should be degraded to a similar extent by the three enzymes.

It has been proposed that one of the basis for the higher hemorrhagic activity of PIII SVMPs, as compared to PI enzymes, has to do with the ability of the former to locate in specific sites in microvasculature of tissues [9,13,18]. This has been demonstrated for the case of jararhagin, a PIII SVMP of the venom of *Bothrops jararaca*, where selective binding to microvessels and a pattern of co-localization of jararhagin and type IV collagen was described [8]. This selective binding is likely to depend on exosites located in the Dis-like and Cys-rich domains of PIII SVMPs. *In vitro* studies have demonstrated that PIII SVMPs selectively bind to proteins containing von Willebrand factor (vWF) A domains, such as vWF, fibrillar-associated collagens with interrupted triple helices (FACITs) and matrylins. Such interaction occurs between these proteins and sequences located in the Cys-rich domain of PIII SVMPs [8,11–14]. In addition, the Dis-like domain of jararhagin might contain sequences that mediate its binding with different types of collagen [9].

Our observations on the tissue localization of the three SVMPs in an ex vivo model conclusively demonstrate, using a quantitatively morphometric approach, the different pattern of distribution of PI and PIII SVMPs, since the former shows a more widespread pattern, whereas the latter preferentially bind to the microvessels and clearly co-localizes with type IV collagen. In addition, our study shows, for the first time, that a PII SVMP presents a pattern of distribution in the tissue highly similar to that of PIII enzymes, i.e. in the microvessels and co-localizing with type IV collagen. Hence, the dimeric PII SVMP, containing only metalloproteinase and disintegrin domains, is preferentially located in the microvasculature. This is reasonable given the high hemorrhagic activity of this PII SVMP [16]. The specific sequences in the PII and PIII enzymes mediating the interaction to microvessels, and the specific sites in the vasculature for interaction with SVMPs remain to be identified. The observed co-localization with type IV collagen does not exclude possible binding to other BM components; however, our findings support the concept that PII and PIII SVMPs preferentially co-localize with the BM. These results support the hypothesis that the high hemorrhagic activity of PII and PIII SVMPs is at least partially due to the selective localization of these enzymes in the BM of microvessels. The co-localization of PII and PIII SVMPs with type IV collagen was observed not only in capillary vessels, but also on arterioles and PCV, thus reflecting the localization of these SVMPs in the BM of the three types of microvessels. The possible pathological effects of SVMPs on these components of the microvasculature, in addition to capillaries, has not been studied and deserve consideration in order to fully understand the vascular pathology in snakebite envenoming.

One puzzling issue has to do with the large variation in hemorrhagic activity between the PII and PIII SVMPs, even though both present a similar pattern of distribution in the micro-vasculature. This finding agrees with previous observations with *Bothrops jararaca* hemorrhagic SVMPs in which there is a great difference in the hemorrhagic potential of PIII SVMPs [10]. These observations suggest that even though the high hemorrhagic activity of PII and PIII SVMPs largely depends on their ability to selectively bind to microvessels, other factors also

determine the hemorrhagic potential of these enzymes. Differences in the turnover rate of hydrolysis of relevant substrates in the BM, especially of type IV collagen, may play a key role. Alternatively, PII and PIII SVMPs might present differences in the exosites in the Dis, Dis-like and Cys-rich domains, which determine subtle variations in the localization of these enzymes in the relevant substrates, with the consequent functional effects related to the proteolysis-induced mechanical destabilization of BM structure. Another possible explanation has to do with differences in the stability of these enzymes in the tissues, with more stable enzymes exerting a higher hemorrhagic effect. This subject deserves further investigation.

Since the hemorrhagic activity of SVMPs is likely to depend on the hydrolysis of BM components [8,18,22], particular attention was placed in this work to the analysis of degradation of BM proteins. Proteomics analysis of exudate collected in the vicinity of the hemorrhagic areas only detected perlecan and nidogen, and no differences were observed between the three SVMP classes regarding BM proteins. No protein fragments of laminin and type IV collagen were detected in this analysis. However, more sensitive immunochemical assessment of skin and exudates revealed subtle variations which might shed light on the mechanisms of hemorrhagic activity. Different patterns of hydrolysis were observed regarding nidogen, laminin and type VI collagen. In contrast, there were evident similarities between the three enzymes concerning hydrolysis of type IV collagen. This has interesting implications because a previous study identified type IV collagen as a likely candidate to play a key role in the onset of hemorrhagic activity, since hemorrhagic and non-hemorrhagic SVMPs differ in the extent of hydrolysis of this collagen [22]. Our present findings are therefore compatible with the hypothesis that degradation of type IV collagen is critical for microvessel damage and hemorrhage. This in turn agrees with the known role of this type of collagen in the mechanical stability of the BM [24-27,38], mostly owing to the presence of a covalently-linked network formed by this BM component [24]. Moreover, a genetic disease associated with mutations in the COL4A1 gene and reduction in the expression of al subunit are associated with pathological alterations in microvessels and hemorrhage in mice, and have been linked to hemorrhagic stroke in humans [39]. In contrast to type IV collagen, hydrolysis of nidogen, laminin and type VI collagen by the three SVMPs showed differences both in the degradation patterns and in the intensity of the bands observed by immunoblotting of exudates, where the PI enzyme showed a more extensive degradation of these substrates. These observations, in the context of a similar extent of hemorrhage by the three SVMPs, suggest that the hydrolysis of nidogen, laminin and type VI collagen might not be directly associated with the onset of microvessel damage leading to hemorrhage but rather may be a general by-product of microvessel damage.

On the other hand, there were notorious differences in the amounts of other ECM proteins, which are not BM components, in the proteomic analysis of exudates collected from mice injected with the three toxins. In general, PI SVMP induced the appearance of higher amounts of various ECM proteins in exudates. This observation may be due to two factors: since a higher absolute amount of this enzyme was injected, owing to its lower hemorrhagic activity, there was a higher proteolytic activity in the tissue, thus resulting in higher hydrolysis. The higher extent of hydrolysis in the case of the PI SVMP observed by immunoblotting of exudates supports this hypothesis. On the other hand, the presence of exosites in PII and PIII SVMPs may contribute to their localization at specific targets in the ECM and on cell membranes, thus reducing the probability of these enzymes to act in a widespread fashion on ECM components. In contrast, the PI SVMP, being devoid of such exosites, would have less restriction to hydrolyze an ample spectrum of ECM substrates, as observed in our proteomics results.

As would be expected, the majority of ECM proteins detected in exudates corresponded to proteolytic fragments, on the basis of their molecular mass. The different pattern of hydrolysis by the SVMP of various ECM components, as detected by Western blot analysis, might be due to variations in the cleavage site preferences among these enzymes. Alternatively, this might depend on the presence of exosites in the non-metalloproteinase domains, which target these enzymes to different substrates in the ECM including BM or to different sequences in particular substrates, as observed by Serrano et al. [12,13].

In addition to ECM proteins, proteomic analysis of exudates allows the detection of serum, intracellular and membrane-associated proteins, and these findings may shed light on the pathological action of SVMPs from a broader perspective. Similar quantitative patterns of plasmaderived proteins were detected in exudates from mice injected with the three toxins. This seems logical, as the presence of these proteins is largely a consequence of overt microvessel damage by the action of these enzymes. Hence, extravasation of blood results in similar amounts of serum plasma proteins, and of hemoglobin as well. An exception to this general trend was observed with hydrolysis products of fibrinogen, which were in higher amounts in exudates from animals injected with the PI SVMP. Since this enzyme has fibrinolytic activity [30] and was injected in a higher dose than the other two SVMPs, this may have resulted in hydrolysis of the fibrin formed as a consequence of extravasation and clot formation.

When the amounts of intracellular proteins were compared in exudates, it was of interest that the PI SVMP, and also the PII SVMP, induced a higher amount of keratins than PIII SVMP. It is suggested that this reflects the ability of the former SVMPs to induce dermonecrosis and blistering which has been shown to be the case with this particular PI SVMP [40], but has not been previously explored for the PII SVMP. It is noteworthy that the dermotoxic action of the PII SVMP occurs when injected at a very low dose. On the other hand, there were several other intracellular proteins whose amounts were higher in exudates collected from mice injected with PII and PIII SVMPs. This suggests that these enzymes induce a higher cytotoxic activity in various cell types in the tissue. The three SVMPs induced a similar extent of skeletal muscle damage, as revealed by the similar amounts of the cytosolic muscle cell marker creatine kinase in exudates; it has been suggested that hemorrhagic SVMPs induce myotoxicity as a consequence of tissue ischemia [41]. The higher amounts of several intracellular markers in exudates collected from PII and PIII SVMPs-injected mice may be due to the targeting of these enzymes, through exosites present in the additional domains, to sites in the plasma membrane or in the vicinity of cells, a hypothesis that remains to be investigated.

In conclusion, our findings demonstrate that PII and PIII hemorrhagic SVMPs co-localize with type IV collagen in capillaries, PCVs and arterioles, whereas PI SVMP presents a more widespread localization in the tissue. This difference in tissue localization is likely to be one of the main reasons behind the higher hemorrhagic activity characteristic of PII and PIII SVMPs, as compared to enzymes of the PI class. Furthermore, immunochemical results support the hypothesis that hydrolysis of type IV collagen is likely to be a key event in SVMP-induced microvessel damage and destabilization leading to hemorrhage.

Supporting Information

S1 Table. List of all proteins identified in wound exudates collected from mice injected with PI (*B. asper*), PII (*B. lateralis*) and PIII (*C. simus*) SVMPs. (PDF)

S2 Table. Serum proteins identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs. (PDF)

S3 Table. Coagulation factors identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs.

(PDF)

S4 Table. Serum proteinase inhibitors identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs. (PDF)

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S5 Table. Intracellular proteins identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs.

(PDF)

S6 Table. Keratins identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs.

(PDF)

S7 Table. Membrane proteins identified in wound exudates collected from mice injected with PI, PII or PIII SVMPs. (PDF)

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Author Contributions

Conceived and designed the experiments: CH TE MBV AR JJC LS SN JMG JWF. Performed the experiments: CH TE MBV AR DM JKAM JJC LS. Analyzed the data: CH TE MBV AR DM JKAM JJC LS SN JMG JWF. Contributed reagents/materials/analysis tools: TE MBV AR JJC SN JMG JWF. Wrote the paper: CH TE MBV AR JJC LS SN JMG JWF.

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ARTICULO II





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Effects of PI and PIII Snake Venom Haemorrhagic Metalloproteinases on the Microvasculature: A Confocal Microscopy Study on the Mouse Cremaster Muscle

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Abstract

The precise mechanisms by which Snake Venom Metalloproteinases (SVMPs) disrupt the microvasculature and cause haemorrhage have not been completely elucidated, and novel in vivo models are needed. In the present study, we compared the effects induced by BaP1, a PI SVMP isolated from Bothrops asper venom, and CsH1, a PIII SVMP from Crotalus simus venom, on cremaster muscle microvasculature by topical application of the toxins on isolated tissue (i.e., ex vivo model), and by intra-scrotal administration of the toxins (i.e., in vivo model). The whole tissue was fixed and immunostained to visualize the three components of blood vessels by confocal microscopy. In the ex vivo model, BaP1 was able to degrade type IV collagen and laminin from the BM of microvessels. Moreover, both SVMPs degraded type IV collagen from the BM in capillaries to a higher extent than in PCV and arterioles. CsH1 had a stronger effect on type IV collagen than BaP1. In the in vivo model, the effect of BaP1 on type IV collagen was widespread to the BM of arterioles and PCV. On the other hand, BaP1 was able to disrupt the endothelial barrier in PCV and to increase vascular permeability. Moreover, this toxin increased the size of gaps between pericytes in PCV and created new gaps between smooth muscle cells in arterioles in ex vivo conditions. These effects were not observed in the case of CsH1. In conclusion, our findings demonstrate that both SVMPs degrade type IV collagen from the BM in capillaries in vivo. Moreover, while the action of CsH1 is more directed to the BM of microvessels, the effects of BaP1 are widespread to other microvascular components. This study provides new insights in the mechanism of haemorrhage and other pathological effects induced by these toxins.



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Introduction

Viperid snakebite envenomings are characterized by drastic alterations in the microvasculature, which cause local and systemic haemorrhage and alterations in tissue regenerative processes [1,2]. Zinc-dependent snake venom metalloproteinases (SVMPs) are largely responsible for these effects, being abundant components in viperid snake venoms [3]. In addition, they are also involved in the pathogenesis of other aspects of local tissue alterations, such as myonecrosis, blister formation, inflammation, and oedema [4].

SVMPs have been classified in three groups according to their domain structure: 1) PI, which comprise only the metalloproteinase domain; 2) PII, which have, in addition to catalytic domain, a disintegrin domain; and 3) PIII, which present a catalytic domain followed by a disintegrin-like domain and a cysteine-rich domain [5]. In general, PIII SVMPs have higher haemorrhagic activity than PI SVMPs. Moreover, differences in the tissue distribution between haemorrhagic PI and PIII SVMPs have been described [6,7], which could have implications in their capacity to induce microvascular damage and haemorrhage.

It has been proposed that the mechanism by which SVMPs disrupt the microvasculature is by hydrolyzing basement membrane (BM) and other extracellular matrix components, thus causing weakening of the mechanical stability of capillaries, and subsequent loss of endothelial cells integrity and extravasation of blood components due to the action of hemodynamic biophysical forces operating in the microvasculature [8,9]. However, the precise mechanism by which SVMPs disrupt the microvasculature, and whether there are differences in the actions between the different types of SVMPs that could explain variations in their haemorrhagic activity, have not been completely elucidated. Moreover, it has been described that extravasation in venules due to the prominent inflammatory reaction characteristic of these envenomings may also contribute to the haemorrhagic mechanism [10–12]. There is very little information on the action of haemorrhagic SVMPs on the various types of vessels in the microvasculature, i.e. capillaries, venules and arterioles.

Several studies have demonstrated the ability of haemorrhagic SVMPs to hydrolyze proteins of the BM and other extracellular components *in vitro* [7,13–17]. In contrast, *in vivo* studies on the action of these toxins are scarce. Previous investigations have used three methodological approaches for assessing the action of SVMPs *in vivo*: immunohistochemistry in tissue sections, immunodetection by Western blot in tissue homogenates or in exudates, and proteomics analysis of wound exudates collected in the vicinity of affected tissue [6,7,16–18]. Despite the relevant contribution of these studies to the understanding of the mechanism(s) of action of SVMPs, in most of them the nature of BM components and their fragments is unknown. Moreover, these methodologies have not allowed a differential analysis of SVMPs effects on the various blood vessel types in the whole tissue. Therefore, there is a need for novel *in vivo* models to study the effects of SVMSs on the different components of the microvasculature using a more detailed and quantitative approach that could complement previous investigations and provide a more comprehensive picture of this relevant pathology.

Due to the transparency and thinness of the cremaster muscle, it is a highly convenient tissue to analyze histological changes by light microscopy. Previous studies have used this muscle to investigate the effects of snake venoms and isolated toxins on the microvasculature by intravital microscopy with low resolution techniques, which allow the observation of venom- and SVMP- induced haemorrhage [19–21]. The use of cremaster muscle for confocal microscopy allows the collection of high-resolution images in three dimensions of longitudinal blood vessels in whole tissue preparations, thus enabling a more detailed and quantitative analysis of microvascular components. In the present study, we compared the effects of two haemorrhagic SVMPs: BaP1, a PI from *Bothrops asper* venom, considered a weak haemorrhagic toxin; and CsH1, a PIII from *Crotalus simus* venom that has a higher haemorrhagic activity. The action of these SVMPs was studied on the three components of blood vessels, i.e. BM, endothelial cells, and smooth muscle cells/ pericytes of the cremaster muscle microvasculature, using an immunofluorescence approach by confocal microscopy. In addition, the role of blood flow and the differential effects of SVMPs on the three vessel types: capillaries, venules and arterioles, were studied. Our findings demonstrate differences in the ability of both SVMPs to degrade type IV collagen in the presence or absence of blood flow, and between the different vessel types. Moreover, differences were observed in the action of these SVMPs on endothelial cell-cell junctions, and on smooth muscle cells and pericytes. This study provides new insights in the mechanism of action of haemorrhagic SVPMs, and describes for the first time novel effects of SVMPs to various components of the microvasculature.

Materials and Methods

Isolation of SVMPs

The PI SVMP BaP1 was isolated from the venom of *Bothrops asper*, as described by Gutiérrez et al. [22] and Watanabe et al. [23], by a combination of ion-exchange chromatography on CM-Sepharose, followed by affinity chromatography on Affi-gel Blue. The PIII SVMP CsH1 was isolated from *Crotalus simus* venom, as described by Herrera et al. [7], by ion-exchange chromatography on DEAE-Sepharose, followed by gel filtration on a Superdex TM 200 10/ 300GL (GE Healthcare, LifeSciences) column (10 x 300 mm) using an ÄKTA FPLC (GE Healthcare, Life Sciences). Homogeneity of SVMP preparations was assessed by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE). Both toxins were isolated from the venoms of more than 40 adult specimens of each species collected in Costa Rica and maintained at the serpentarium of Instituto Clodomiro Picado, Costa Rica. After collection, venoms of each species were separately pooled, lyophilized, and stored at -20°C until used. The Minimum Hemorrhagic Dose (MHD), corresponding to the amount of enzyme that induces a hemorrhagic spot of 10 mm diameter in mice 2 h after injection, is 20 µg for BaP1 [22] and 2.2 µg for CsH1 [7].

Ethics statement

Inbred male C57BL/6 mice (20-25 g body weight) were purchased from Charles River Laboratories, Cambridge, UK and Laboratorio de Ensayos Biológicos, LEBI, Costa Rica. The protocols involving the use of animals were approved by the Animal Welfare and Ethical Review Board (AWERB), Queen Mary University of London, and the Institutional Committee for the Care and Use of Laboratory Animals (CICUA), University of Costa Rica, and meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS) and UK legislation for the protection of animals. Mice were maintained under standard conditions of temperature (22±2°C), light/dark cycles of 12 h, and food and water *ad libitum*.

Ex vivo effects of SVMPs on cremaster muscle vasculature (model without blood flow)

Groups of four mice were sacrificed by cervical dislocation and the cremaster muscle was dissected out. The isolated muscles were incubated, at room temperature, with either BaP1 or CsH1 SVMPs dissolved in 100 μ L of 0.12 M NaCl, 0.04 M phosphate, pH 7.2 solution (PBS). Initially, three doses of BaP1 (10, 30 and 100 μ g) and two incubation times (5 and 15 min) were evaluated in order to study the dose and time dependence of the effects. The dose of 30 µg of BaP1 and the incubation time of 15 min were selected for further studies. In the case of CsH1, a dose of 15 µg was selected as to induce a haemorrhage of similar intensity in the cremaster muscle to that caused by 30 µg of BaP1 by intravital microscopy, as described previously [21]. This 2:1 mass ratio of the SVMPs corresponds to an approximate molar ratio of 5:1. Control tissues were incubated with PBS alone. After incubation, tissues were washed three times with PBS, fixed and immunostained for observation by confocal microscopy, as described below.

In vivo effects of SVMPs on cremaster muscle vasculature (model with blood flow)

Groups of four mice were anesthetized with ketamine/xylazine and injected via the intrascrotal (i.s.) route with either 60 μ g of BaP1 or 30 μ g of CsH1, dissolved in 300 μ l of PBS. These doses correspond to twice the dose used in the *ex vivo* model since each mouse has two cremaster muscles. PBS (300 μ l) was used as control. Fifteen min after i.s. injection, mice were sacrificed by cervical dislocation and the cremaster muscles were dissected out, washed three times with PBS, fixed, and immunostained for observation by confocal microscopy, as described below.

Immunostaining of mouse cremaster muscles

Tissues were fixed with 4% paraformaldehyde in PBS (for collagen IV and nidogen immunostaining) or methanol (for laminin immunostaining) for 30 min at 4°C. Whole tissues were incubated for 4 h at room temperature in blocking and permeabilization solution (12.5% goat serum, 12.5% fetal bovine serum, 0.5% Triton X-100 in PBS) under stirring at room temperature. Then, the tissues were incubated for 48 h at 4°C with either rabbit anti-collagen type IV polyclonal antibody (Abcam ab19808) at a dilution of 1:100, rabbit anti-nidogen 1 polyclonal antibody (Abcam ab14511) at a dilution of 1:200, or rabbit anti-laminin polyclonal antibody (Abcam ab11575) at a dilution of 1:100, to visualize the vascular BM. Simultaneously, the tissues were incubated with Cy3-labeled mouse anti-actin a smooth muscle monoclonal antibody (clone 1A4, Sigma C6198), at a dilution of 1:200, and anti-mouse vascular endothelial (VE) cadherin monoclonal antibody (clone BV14, eBioscience 14-1442), at a dilution of 1:200, to visualize the smooth muscle/pericytes and endothelial cell-to-cell junctions, respectively. The anti-VE cadherin was previously labeled with Alexa 647 according to the Alexa Fluor 647 * Monoclonal Antibody Labeling Kit (Molecular Probes A20186). After 48 h of incubation, tissues were washed with PBS and incubated for 4 h at 4°C with goat anti-rabbit polyclonal antibody (Invitrogen A11034). All the antibodies were diluted in PBS containing 10% fetal bovine serum. After 4 h of incubation, tissues were washed with PBS and the whole tissues were mounted on glass slides in PBS for confocal microscopy observation, as described in the next section.

Analysis of tissues by confocal microscopy

Immunostained tissues were visualized using a Zeiss LSM 5 Pascal laser-scanning confocal microscope (Carl Zeiss Ltd) incorporating a 40X water objective (numerical aperture 0.8), and an Olympus Fluoview FV1000 laser-scanning confocal microscope incorporating a 40X oil objective (numerical aperture 1.3). Three-dimensional images of post-capillary venules (PCV), arterioles and capillaries in the tissue were acquired with sequential scanning of different channels at a resolution of $1,024 \times 1,024$ dpi, corresponding to a voxel size of $0.22 \times 0.22 \times 1.18 \,\mu\text{m}$ and $0.31 \times 0.31 \times 0.57 \,\mu\text{m}$ in the X × Y × Z plans, with the Zeiss and Olympus confocal microscope, respectively. Images of treated tissues were taken using the same settings used for

control tissues for each experiment. At least five images of PCV and arterioles (20–45 μ m diameter and 200 μ m length) were obtained from the middle along the longitudinal axis until the lateral top segment of the vessel (i.e. semi-vessel) per tissue, whereas at least eight images of whole capillaries (200 μ m length) were collected per tissue. Analysis of total fluorescence intensity (average gray value of all the pixels in the analyzed area), and size and density of gaps between adjacent smooth muscle/pericytes were carried out using Image J software. Three-dimensional reconstitution of the images was carried out using IMARIS x64 7.4.2 software.

In vivo vascular permeability assay

To evaluate vascular permeability induced by SVMPs in the skin of mice, Evans Blue dye was used as marker of extravasation according to the protocol described by Radu and Chernoff [24], with some modifications. Briefly, groups of five animals (18–20 g) were injected by intravenous (i.v.) route in the lateral tail vein with 200 μ l of Evans Blue dye (6 mg/ml), dissolved in PBS. After 5 min, mice were injected by intradermal (i.d.) route in the ventral abdominal region with either 2 μ g of BaP1 or 1 μ g of CsH1, dissolved in 50 μ l of PBS. These doses were selected since they do not induce haemorrhage in the skin, as to avoid the development of a haemorrhagic lesion that would interfere with the measurement of Evans Blue extravasation, and in order to maintain the same protein mass ratio (2:1) used in the *in vivo* model described above. Controls received 50 μ l of PBS alone by i.d. route. Fifteen minutes after i.d. administration, mice were sacrificed by cervical dislocation, their skin was removed, and the area of plasma, i.e. Evans Blue, extravasation was measured.

Statistical analysis

Results were expressed as the mean ± standard error of mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) and multifactorial ANOVA (when more than one factor was analyzed) with Tukey as *post hoc* test using the IBM SPSS Statistics 22.0 and GraphPad Prim 6 software.

Results

Effects on BM components

In order to study the dose and time dependence of the effects induced by BaP1 on the vasculature of cremaster muscle, and to define the optimal conditions for further studies, isolated tissues were incubated with different amounts of BaP1 for either 5 or 15 min. In conditions without blood flow and topical application of the toxin, BaP1 induced a decrease in fluorescence intensity of type IV collagen immunostaining of the BM of capillaries, as compared to controls, at multiple doses and two time points (Fig 1B). No significant differences were observed in the fluorescence intensity of type IV collagen of arterioles (Fig 1A) and PCV (Fig 1C); however, there was a trend to a dose-dependent decrease of the intensity in PCV.

The intermediate dose (i.e. 30 µg) and the time lapse of 15 min were selected to study the effect of BaP1 on other BM components in the *ex vivo* model. Under these conditions, BaP1 induced a decrease in fluorescence intensity for laminin at the BM of capillaries (p < 0.05) and PCV (p < 0.01), but not in arterioles (Fig 2A and 2C). Conversely, no significant differences in fluorescence intensity were observed in vascular BM immunostained for nidogen (Fig 2B).

In order to study the effects of BaP1 on vascular BM in conditions of blood flow, this SVMP was injected via the i.s. route to anesthetized animals. After 15 min, the tissues were dissected out for analysis. Type IV collagen was selected for these studies since it plays an important structural role in the mechanical stability of BM [25-30], and has been identified as a potential

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Fig 1. Dose and time dependency of BaP1 effects on type IV collagen from vascular BM on isolated mouse cremaster muscle. Isolated cremaster muscles were incubated with different amounts of BaP1 (10, 30 and 100 µg) for either 5 or 15 min (model without blood flow). Control tissues were incubated with PBS. Whole tissues were fixed and immunostained for observation by confocal microscopy and analysis of total fluorescence intensity for type IV collagen. Results are expressed as the mean ± SEM of the percentage of intensity related to control of at least five images of each vessel type: (A) arterioles, (B) capillaries, and (C) PCV per cremaster (n = 4). Below each graph, representative three-dimensional images of each vessel type immunostained for type IV collagen are shown with a gray color coding spectrum (black as low fluorescence intensity regions and white as high fluorescence intensity regions) for BaP1 (30 µg) and control at 15 min. The images show a decrease in fluorescence intensity for type IV collage in BM of capillaries of treated tissues as compared to control, whereas no significant reduction was observed in arterioles and PCV. Scale bar represents 30 µm. *p < 0.05, **p < 0.001 as compared to control, control. C: control; Col IV: type IV collagen; PCV: post-capillary venules.

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key target in the action of haemorrhagic SVMPs [6,7,17,31]. In contrast to the effect induced by BaP1 for BM type IV collagen in the *ex vivo* model, which occurred predominantly in capillaries (Fig 3A), this SVMP induced a widespread reduction in immunostaining in arterioles and PCV in the *in vivo* model, i.e. in conditions in which blood flow was present (Fig 3B).

In contrast to BaP1, the PIII SVMP CsH1 induced a decrease in fluorescence intensity for type IV collagen immunostaining in the three vessels types in the isolated tissue, i.e. without blood flow (Fig 3C), as well as in conditions in which flow was present (Figs 3D and 2E). The overall multivariate analysis of variance for the effect of BaP1 and CsH1 in the *ex vivo* and *in vivo* models highlights a difference between treatments (p < 0.001) and vessel types (p < 0.05), but not between presence or absence of blood flow.

Effects on endothelial cell-to-cell junctions

Antibodies against VE cadherin were used as markers to evaluate the effects of BaP1 and CsH1 on the endothelial cell-to-cell junctions of blood vessels. When SVMPs were evaluated *ex vivo* on the isolated mouse cremaster muscle, no significant changes were observed in total fluorescence intensity for VE cadherin staining or vascular endothelial morphology, as compared to controls. However, when effects were evaluated *in vivo*, a disruption in the alignment of VE-cadherin staining was observed in 50% of the analyzed images of PCV in tissues treated with BaP1, as compared to controls (Fig 4). In spite of this loss of continuous staining induced by

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Fig 2. Effect of BaP1 on laminin and nidogen from vascular BM on isotated mouse cremaster muscle. Isolated cremaster muscles were incubated with 30 µg of BaP1 for 15 min (model without blood flow). Control tissues were incubated with PBS. Whole tissues were fixed and immunostained for observation by confocal microscopy and analysis of total fluorescence intensity for (A) laminin and (B) nidogen. Results are expressed as the mean \pm SEM of the percentage of intensity related to control of at least five images of each vessel type per cremaster (n = 4). (C) Representative three-dimensional images of each vessel type immunostained for laminin are shown with a gray color coding spectrum (black as low fluorescence intensity regions and white as high fluorescence intensity regions). The images show a decrease in fluorescence intensity for laminin in BM of capillaries and PCV of treated tissues as compared to control, whereas no reduction in the fluorescence intensity was observed for nidogen. Scale bar represents 30 µm. *p < 0.05, **p < 0.001 as compared to control. Lam: laminin; Nid: nidogen; PCV: post-capillary venules.

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BaP1, no significant decrease in total fluorescence intensity for VE cadherin staining was found in PCV in the *in vivo* model. This change in morphology was neither observed in arterioles nor capillaries after treatment with BaP1. In contrast, CsH1 did not induce any evident change in vascular endothelial morphology in any of the studied models.

Effects on vascular permeability

In order to evaluate vascular permeability induced by both SVMPs, the area of plasma extravasation induced by toxin after i.d. administration was measured using Evans Blue dye as marker. The same protein mass ratio (2:1) used in the immunohistochemistry experiments was employed. An increase of vascular permeability was evident after administration of 2 µg of BaP1 with a mean extravasation area of 87 ± 13 mm² (Eig 5). By contrast, 1 µg of CsH1 did not induce an increment in vascular permeability, since most of the tissues looked similar to controls, with only small areas of extravasation observed in some animals. Control animals did not show areas of extravasation in the skin.

Effects on gaps between adjacent smooth muscle cells and pericytes

When BaP1 was applied directly on isolated mouse cremaster muscles (model without blood flow), a significant increase on the size of gaps between adjacent smooth muscle cells and pericytes was observed in arterioles (p < 0.001, Fig 6A) and PCV (p < 0.05, Fig 6B), as compared to controls. Moreover, this increment in gap size was observed together with an increase in

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gap density in arterioles (p < 0.05, Fig 6C), but not in PCV (Fig 6D). Conversely, when BaP1 was administered in the anesthetized animal (model with blood flow), no changes were observed in gap size and density for neither arterioles nor PCV. Furthermore, CsH1 did not induce any change in size and density of gaps in neither arterioles nor PCV in any of the studied models. The overall multivariate analysis of variance for the effect of BaP1 and CsH1 in the *ex viro* and *in vivo* models indicated a difference between treatments (p < 0.01) and between conditions of presence and absence of blood flow (p < 0.01) for both arterioles and PCV.



Fig 4. In vivo effect of BaP1 on endothelial cell-to-cell junctions on mouse cremaster muscle vasculature. Anesthetized mice were injected by intrascrotal route with 60 µg of BaP1 (model with blood flow). PBS was injected in controls. After 15 min, cremaster muscles were dissected out, fixed and immunostained for observation by confocal microscopy and analysis of the endothelial cell-to-cell junctions. Figure shows representative three-dimensional images from at least five images of each vessel type per animal (n = 4) immunostained for VEcadherin. Notice the loss of junctional VE cadherin staining in PCV of treated tissues (arrows). Scale bar represents 30 µm. Art: arterioles; Cap: capillaries; PCV: post-capillary venules.

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Discussion

This study assessed the action of two hemorrhagic SVMPs on the three main components of blood vessels in the microvasculature. Our observations clearly underscore that these SVMPs alter BM components in microvessels, particularly in capillaries. Previous studies have demonstrated the ability of these and other SVMPs to degrade type IV collagen, laminin and nidogen in different *in vivo* models [7,16–18,32]. However, since most of these studies used tissue homogenates and wound exudates, the precise origin of the degradation products detected is unknown. Our work demonstrates a decrease in the immunostaining of type IV collagen and laminin from the BM of blood vessels in a whole tissue preparation, as detected by confocal microscopy, suggesting a degradation of BM components by these enzymes.

The action of SVMPs on the vascular BM depends on their ability to reach this extracellular matrix structure, and to bind and degrade specific BM components. Moreover, the diffusion of degraded proteins and fragments away from the BM after hydrolysis also influences the immunostaining of these components in tissue preparations. Previous studies have demonstrated that PIII SVMPs co-localize with BM components of blood vessels to a higher extent than PI SVMPs, which have a more widespread distribution in the tissue [6,7]. This higher binding capacity of PIII SVMPs to microvessels is associated with the presence of exosites located in the Dis-like and Cys-rich domains of the toxin [6,33–37]. This could explain the greater effect of PIII SVMPs as compared to PI SVMP on the vascular BM on isolated tissue.

According to our results, both PI and PIII SVMPs degrade type IV collagen from the BM in capillaries to a higher extent than in PCV and arterioles. Moreover, it seems that the BM of PCV is more susceptible to degradation by SVMPs than its arteriolar counterpart. This might suggest that the ability of SVMPs to reach BM of PCV and arterioles is limited due to structural constraints in the wall of these vessels. However, BaP1 was able to degrade laminin in PCV, evidencing its ability to reach the BM of this vessel type. Moreover, it has been demonstrated that CsH1 binds to BM components of arterioles, capillaries, and PCV to a similar extent [7]. Taken together, these observations argue against the poor accessibility as the main cause behind the differences in the patterns of degradation of BM in various microvessel types.

The ability of BaP1 to reduce the immunostaining of laminin to a greater extent than type IV collagen might be due to the fact that type IV collagen constitutes a more stable covalently-





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linked network, in contrast to laminin [25–30]. Hence, it is likely that, even if type IV collagen is hydrolyzed, epitopes could remain within the BM, whereas laminin degradation products might be easily washed out from this structure. Previous investigations demonstrated the degradation of nidogen by SVMPs on tissue homogenates and on the BM preparation Matrigel *in vitro* [7,16,17,32]. Conversely, we did not observe a reduction in the immunostaining of nidogen, which may indicate that BaP1 does not degrade nidogen of vascular BM on the isolated cremaster muscle. One explanation for this disagreement is that epitopes could remain bound to BM even after nidogen is hydrolyzed by BaP1, which would be evidenced by western blot of tissue homogenates but not by immunohistochemistry. Another possible explanation is that nidogen and its degradation products detected in western blot of tissue homogenates might come from the BM of other tissue structures such as muscle and nerves.

When toxins were evaluated in the *in vivo* model, i.e. in the presence of blood flow, the effect of CsH1 on type IV collagen of blood vessels was similar to the effect seen in the *cx vivo* model. However, in the case of BaP1, loss of immunostaining was also evident in the BM of arterioles and PCV in the presence of blood flow, but not in its absence. This discrepancy is

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Fig 6. Effect of BaP1 and CsH1 on size and density of gaps between adjacent smooth muscle and pericytes on mouse cremaster muscle vasculature. Isolated cremaster muscles were incubated with either 30 µg of BaP1 or 15 µg of CsH1 (model without blood flow). In another experiment, anesthetized mice were injected by intrascrotal route with either 60 µg of BaP1 or 30 µg of CsH1 (model with blood flow). Controls were incubated or injected with PBS. After 15 min of exposition to toxin in each model, whole cremaster muscles were fixed and immunostained for observation by confocal microscopy and analysis of the gaps between adjacent smooth muscle and pericytes. Results are expressed as the mean \pm SEM of the (A, B) gap size and (C, D) gap density (number of gaps per vessel area) of at least five images of arterioles and PCV per cremaster (n = 4). (E) Representative three-dimensional images of each vessel type immunostained for actin α smooth muscle are shown for BaP1 30 µg and control in the model without blood flow. Notice the increase in the gap size (arrows) in arterioles and PCV of treated tissues as compared to control. Scale bar represents 30 µm. *p < 0.05, **p < 0.001 as compared to control. C: control; PCV: post-capillary venules.

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likely due to the different distribution of toxins in the tissue in these two experimental settings. In the *ex vivo* model, toxins were topically applied on the isolated tissue, i.e. in absence of blood flow. Instead, in the *in vivo* model SVMPs were applied by the i.s. route, in the presence of blood flow. Thus, the toxin distribution is likely to be different in these experimental models. Differences in temperature might also affect the distribution and enzymatic activity of the SVMPs, since the *ex vivo* model is performed at room temperature, whereas *in vivo* the toxins are acting at body temperature. On the other hand, it has been described that the mechanical properties of the BM vary depending on the action of hemodynamic biophysical forces (i.e. wall tension and shear stress) operating under blood flow conditions in the microvasculature [38]. Therefore, under blood flow the susceptibility of BM components to the action of proteinases may be higher owing to the increased wall tension and mechanical stress. Studies have demonstrated that wall tension may play role in increasing the expression and activity of matrix metalloproteinases (MMPs) [39–41]. This hypothesis as related to SVMPs deserves further investigation. In addition, degradation products generated as a result of hydrolysis by SVMPs might be easily removed from the tissue *in vivo* whereby draining through the lymphatic vessels operate.

The degradation of type 1V collagen in BM of capillaries induced by both SVMPs, with or without blood flow, supports the hypothesis that hydrolysis of this protein is a key event in the microvascular damage and haemorrhagic action of SVMP, as previously proposed [6,7,17,31]. Moreover, the fact that SVMPs reduce the immunostaining of type 1V collagen in conditions of lack of blood flow lends support to the 'two-step' hypothesis for explaining the mechanism of action of hemorrhagic SVMPs [8]. The first step, i.e. hydrolysis of BM components, which can occur in the absence of flow as shown here, is a separate event from the second step, i.e. the distention and disruption of capillary wall integrity, which depends on blood flow.

Endothelial cells are an important component of vascular vessels. Previous work has shown alterations in vascular endothelial cells induced by haemorrhagic SVMPs, an effect associated *in vivo* with the distention of the capillary wall as a consequence of the hydrolysis and posterior weakening of the BM. This rapid effect *in vivo*, occurring within few min, is not due to a direct cytotoxic effect on endothelial cells [19,21,42–44]. It was therefore of interest to assess the action of SVMPs on endothelial cells in our model.

VE-cadherin is a transmembrane protein exclusively expressed by endothelial cells with an adhesive function in the vascular cell-cell contact. VE cadherin plays an important role in the microvascular integrity [45,46] and contributes to the regulation of vascular permeability [47]. Our results demonstrate that BaP1 is able to disrupt the endothelial barrier in PCV in the *in vivo* model. This change in the morphology induced by BaP1 in cell-cell junctions is similar to that described after an inflammatory stimulus, and is associated with changes in VE-cadherin localization, internalization or disassembly [47–49]. Interestingly, a mechanism of SVMP-induced extravasation, known as haemorrhage *per diapedesis*, has been described in PCV; in this case erythrocyte extravasation occurs through widened intercellular junctions in venular endothelial cells [11]. Thus, the possible effect of SVMPs in endothelial cells junctions in PCV, using VE-cadherin as a marker, may provide clues on the mechanism of haemorrhage *per diapedesis*.

The observed effect of BaP1 on VE-cadherin could be due to proteolysis. However, the fact that such effect occurred in the *in vivo* model, but not in the *ex vivo* setting, and that VE-cadherin would be accessible for hydrolysis in both experimental conditions, argues against this explanation. On the other hand, it was somehow surprising that CsH1 did not exert this effect of VE-cadherin. An explanation for this apparently puzzling observation may have to do with the different pro-inflammatory activity of these toxins, evidenced by their ability to induce extravasation of Evans Blue. BaP1 exerts a strong pro-inflammatory action, causing oedema associated with the synthesis and release of several inflammatory mediators [21,22,50–53]. In contrast, CsH1 exerts a much lower increase in plasma extravasation in our experimental setting at the dose tested. Since increments in vascular permeability are associated with opening of endothelial cell junctions, which in turn involve VE-cadherin, the different effect described for the action of these two SVMPs in this cell marker can be explained on the basis of their different pro-inflammatory activity. This mechanism would operate *in vivo* but not in *ex vivo* conditions where blood flow is absent.

Other important components of the microvasculature are smooth muscle cells and pericytes. In most tissues arterioles are surrounded by layers of smooth muscle cells with tight junctions between adjacent cells, whereas PCV are irregularly covered by smooth muscle cells, and have a net-like cell layer of pericytes embedded within the venular BM, with gaps between adjacent cells [54–56]. The effects of SVMPs on these vascular components have not been studied before and may, therefore, illustrate a hitherto unknown aspect of SVMP-induced local pathology.

Our results demonstrate that BaP1 induces an increase in density of gaps between smooth muscle cells/pericytes in arterioles but not in PCV in *ex vivo* conditions, i.e. without blood flow. Thus, new gaps are created in arterioles, while there is an increase in the size of pre-existing gaps in PCV. On the other hand, in the presence of blood flow these effects on arterioles and PCV were not observed for either toxin.

This increase in gap size in PCV and neo-formation of gaps in arterioles could be associated with cell damage or cell contraction. Previous studies have described an increase in the gap size in PCV after inflammatory stimulus [57–59]. However, when BaP1 was injected in the tissue with blood flow, in conditions where inflammation develops, no effects on gaps were observed, thus arguing against inflammation as the cause of these phenomena. Interestingly, CsH1 did not induce any effect on either gap size and gap density. This could be explained on the basis of the presence of exosites in the extra domains of CsH1, which are likely to direct and concentrate this SVMP in the BM of the vessels. In contrast, BaP1 can operate on different tissues or structures due to the lack of targeting of this SVMP to vessel walls, as described previously [7]. Thus, the different 'directionality' of SVMPs having variable domain structure may impact on their ability to damage capillaries, arterioles and PCV.

Pericytes are closely associated with endothelial cells, and play a role in maintaining the integrity of the vessel walls, vessel contractility, regulation of blood flow, vascular BM remodeling during inflammation, vascular development, angiogenesis, and wound healing [59–62]. Thus, the effects of SVMPs on arteriolar smooth muscle cells and pericytes could play an important role in the pathogenesis of tissue damage and constitute an unexplored target for the search of novel therapeutic avenues in snakebite envenoming. Previous studies have demonstrated that *B. asper* venom affects the smooth muscle of lymphatic vessels through the action of myotoxic phospholipases A₂ [63]; however, our findings on the action of SVMPs on smooth muscle cells and pericytes have not been previously described for a SVMP. In the context of the overall pathogenesis of viperid venom-induced local tissue damage, it is likely that arteriolar and other smooth muscle cells might be affected by the combined action of myotoxic phospholipases A₂ and SVMPs, especially PI SVMP.

Conclusions

The study of the *ex vivo* and *in vivo* effects of SVMPs on whole tissue using high resolution confocal microscopy techniques provides new insights into the effects of SVMPMs on the three components of microvasculature. Our findings demonstrate that both haemorrhagic SVMPs are able to degrade type IV collagen in BM of capillaries *in vivo*, which supports the hypothesis that hydrolysis of this protein is an important event in the haemorrhagic action of these toxins. Moreover, BaP1 disrupts the endothelial barrier in PCV and increases vascular permeability. Furthermore, BaP1 is able to create new gaps between smooth muscle cells in arterioles and increase gap size between pericytes in PCV in *ex vivo* conditions. In contrast, the action of the PIII SVMP CsH1 is more directed towards the BM of microvessels, probably as a consequence of the presence of exosites in various domains of this enzyme, which direct it to

targets in the BM. Our results underscore the complexity of the pathological effects induced by SVMPs in the microvasculature.

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Funding acquisition: MV SN JMG.

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Muscle Tissue Damage Induced by the Venom of *Bothrops asper*: Identification of Early and Late Pathological Events through Proteomic Analysis

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Abstract

The time-course of the pathological effects induced by the venom of the snake Bothrops asper in muscle tissue was investigated by a combination of histology, proteomic analysis of exudates collected in the vicinity of damaged muscle, and Immunodetection of extracellular matrix proteins in exudates. Proteomic assay of exudates has become an excellent new methodological tool to detect key biomarkers of tissue alterations for a more integrative perspective of snake venom-induced pathology. The time-course analysis of the intracellular proteins showed an early presence of cytosolic and mitochondrial proteins in exudates, while cytoskeletal proteins increased later on. This underscores the rapid cytotoxic effect of venom, especially in muscle fibers, due to the action of myotoxic phospholipases A2, followed by the action of proteinases in the cytoskeleton of damaged muscle fibers. Similarly, the early presence of basement membrane (BM) and other extracellular matrix (ECM) proteins in exudates reflects the rapid microvascular damage and hemorrhage induced by snake venom metalloproteinases. The presence of fragments of type IV collagen and perlecan one hour after envenoming suggests that hydrolysis of these mechanically/structurallyrelevant BM components plays a key role in the genesis of hemorrhage. On the other hand, the increment of some ECM proteins in the exudate at later time intervals is likely a consequence of the action of endogenous matrix metalloproteinases (MMPs) or of de novo synthesis of ECM proteins during tissue remodeling as part of the inflammatory reaction. Our results offer relevant insights for a more integrative and systematic understanding of the time-course dynamics of muscle tissue damage induced by B. asper venom and possibly other viperid venoms.
Competing Interests: The authors have declared that no competing interests exist.

Author Summary

The local pathology induced by viperid snakes is characterized by a complex of alterations as consequence of direct and indirect effects of the toxins present in the venom, as well as the host response to tissue damage, and constitutes a dynamic process of degenerative and reparative events. The pathogenesis of local effects induced by Bothrops asper venom has been studied by traditional methodologies. Recently, proteomic analysis of wound exudates collected in the vicinity of affected tissue has become a powerful tool to study the pathogenesis of local envenoming from a more integrative perspective. Thus, in the present study we analyzed the dynamics of the local effects induced by B. asper venom in the gastrocnemius muscle of mice through a proteomic and immunochemistry approach in order to identify biomarkers of tissue damage and repair during the course of envenoming. Our results showed an early presence of cytosolic and mitochondrial proteins in exudates as compared to cytoskeletal proteins, which reflect the rapid cytotoxic effect of venom, followed by the action of endogenous proteinases in the cytoskeleton of damaged muscle fibers later on in the course of envenoming. On the other hand, the early presence of extracellular matrix components and the increment of some of them in exudates, reflect the rapid microvascular damage and hemorrhage induced by the venoni, followed by the action of endogenous matrix metalloproteinases (MMPs) during tissue remodeling as part of the inflammatory response. Overall our study allowed the identification of key biomarkers of tissue damage and repair as part of the pathological effects induced by B. asper venom in skeletal muscle, which offer relevant insights for a better understanding of the complex dynamics of local pathology induced by viperid snakebite envenoming.

Introduction

The viperid snake *Bothrops asper* is responsible for most snakebite cases in Central America and some regions of Mexico and South America [1,2]. The local pathology induced by viperid snakes is characterized by edema, blistering, hemorrhage, lymphatic vessel damage, and necrosis of skin and muscle, some of which can be attributed to the degradation of extracellular matrix (ECM) [1,3]. Such alterations develop very rapidly after the bite, and in some cases can lead to permanent tissue damage, regardless of the application of antivenom treatment. Significant efforts have been undertaken over the last several decades to identify the toxins responsible for these effects, as well as to characterize the pathogenesis of these alterations [3–5]. Nevertheless, the complexity of this pathology demands further analyses into hitherto unknown aspects of tissue damage and the complex interplay between degenerative and early reparative events. As envenoming is a dynamic event, it is critical to investigate the process over time, which is the main focus of this study.

The pathogenesis of local effects induced by *B. asper* venom has been studied by traditional methodologies, such as histological and ultrastructural analyses, immunohistochemical methods, and quantification of particular components and tissue markers in tissue homogenates or fluids, as a consequence of the action of crude venom and purified toxins [3,6-12]. Despite significant advances in the study of local tissue damage with these approaches, subtle changes in key biomarkers of tissue damage and repair during the course of envenoming remain to be identified and characterized. Moreover, since the venom is a highly complex mixture of components functioning over time, relevant information related to synergistic action of toxins could be missed when working only with isolated toxins; therefore, studies with crude venom

may better advance our understanding from a predominantly reductionist to a holistic view of these multifactorial time-dependent phenomena.

Recently, proteomic analysis of exudates collected around the affected tissue has become a new methodological tool to study the pathogenesis of local tissue damage induced by snake venom from a more integrative perspective [13–17]. This approach has been used to study the alterations caused by *B. asper* snake venom [15], and some of its toxins, such as a myotoxic phospholipase A_2 (PLA₂) and a hemorrhagic snake venom metalloproteinase (SVMP) [13,14,16]. Moreover, proteomic analysis has allowed the comparison between the action of different types of hemorrhagic and non-hemorrhagic SVMPs [13,16,17]. These studies have identified differences in the species and abundance of intracellular proteins, ECM components, and other proteins present in exudates, which offer new insights in the mechanism of action of these toxins, and in the tissue damage induced by the venom [13–16]. However, these studies have been carried only at early time periods in the course of envenoming and therefore provide only a narrow window within the whole scenario of local pathology.

In the present study we analyzed the time-course variation in the protein composition and abundance of wound exudates collected from mouse gastrocnemius muscle injected with *B. asper* snake venom utilizing a proteomic and immunochemistry approach, in conjunction with histological analysis of tissue alterations, with the aim of identifying biomarkers of tissue damage and tissue remodeling characteristic of early and late stages of envenoming. This approach allowed the identification of key differences in some intracellular proteins and ECM components over time, which underscores the rapid cytotoxic and hemorrhagic effect of venom, followed by the action of endogenous proteinases associated with tissue remodeling later on in the course of envenoming. These results offer relevant insights for a better understanding of the complex pathological phenomena of viperid snakebite envenoming.

Methods

Venom

B. asper venom was obtained from more than 40 adult specimens collected in the Pacific region of Costa Rica and maintained at the serpentarium of Instituto Clodomiro Picado. After collection, venoms were pooled, lyophilized, and stored at -20°C until used.

Ethics statement

CD-1 mice with a body weight between 18 and 20 g were used for the *in vivo* studies. All the experimental protocols involving the use of animals were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (protocol approval number CICUA 025–15), and meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS).

Histology

Groups of four CD-1 mice (18–20 g) were injected in the right gastrocnemius with 50 μ g of *B. asper* venom, dissolved in 50 μ L of 0.12 M NaCl, 0.04 M phosphate, pH 7.2 solution (PBS). Control mice were injected with PBS alone. After 1, 6 and 24 h of injection, mice were sacrificed by CO₂ inhalation and samples of the injected muscles were resected and added to 10% formalin solution in PBS. After 48 h fixation, routine processing of tissues was performed, followed by embedding in paraffin. Sections of 5 μ m thickness were obtained for each sample and stained with hematoxylin–eosin for light microscopic observation.

Collection of wound exudates

Groups of five CD-1 mice (18–20 g) were injected in the right gastrocnemius with 50 µg of *B.* asper venom, dissolved in 50 µL of PBS. After 1, 6 and 24 h of injection, mice were sacrificed by CO₂ inhalation, a 5 mm incision was made with a scalpel in the skin overlying the injected muscle, and a heparinized capillary tube was introduced under the skin to collect the wound exudate fluid. An approximate volume of 20–50 µL of exudate was collected from each mouse. Exudate samples were then pooled and lyophilized for further analysis.

Quantification of creatine kinase (CK) activity in wound exudates

Wound exudates were collected as previously described and centrifuged at 5000 g for 3 min. The CK activity of supernatants was determined using a commercial kit (CK-Nac, Biocon Diagnostik, Germany). CK activity was expressed in International Units /L (IU/L).

Proteomic analysis of wound exudates

Lyophilized wound exudate samples were resuspended in water and protein quantification was performed using micro BCA protein assay kit (Thermo Scientific). Twenty micrograms of protein was precipitated with acetone, resuspended in Laemmli buffer and separated in a 5–20% precast electrophoresis gel (Bio-Rad). The gel was stained with Coomassie Brilliant Blue and lanes were cut into 8 equal size slices. Gel slices were destained for 3 h and the proteins were reduced (10 mM dithiothreitol, DTT) and alkylated (50 mM iodoacetamide) at room temperature. Gel slices were then washed with 100 mM ammonium bicarbonate, dehydrated with acetonitrile and dried in a speed vac, followed by in-gel digestion with a solution of Promega modified trypsin (20 ng/ μ L) in 50 mM ammonium bicarbonate for 30 min on ice. Excess trypsin solution was removed and the digestion continued for 18 h at 37°C. The resulting tryptic peptides were extracted from gel slices with two 30 μ L aliquots of a 50% acetonitrile/5% formic acid solution. These extracts were combined and dried to 15 μ L for mass spectrometric (MS) analysis.

LC/MS/MS was performed using a Thermo Electron Orbitrap Velos ETD mass spectrometer system. Analytical columns were fabricated in-house by packing 0.5 cm of irregular C18 Beads (YMC Gel ODS-A, 12 nm, I-10-25 um) followed by 7.5 cm Jupiter 10 μ m C18 packing material (Phenomenex, Torrance, CA) into 360 x 75 μ m fused silica (Polymicro Technologies, Phoenix, AZ) behind a bottleneck. Samples were loaded directly onto these columns for the C18 analytical runs. 7 μ L of the extract was injected, and the peptides were eluted from the column at 0.5 μ L/min using an acetonitrile/0.1M acetic acid gradient (2–90% acetonitrile over 1 h). The instrument was set to Full MS (m/z 300–1600) resolution of 60,000 and programmed to acquire a cycle of one mass spectrum followed by collision-induced dissociation (CID) MS/MS performed in the ion trap on the twenty most abundant ions in a data-dependent mode. Dynamic exclusion was enabled with an exclusion list of 400 masses, duration of 60 seconds, and repeat count of 1. The electrospray voltage was set to 2.4 kV, and the capillary temperature was 265°C.

The data were analyzed by database searching using the Sequest search algorithm in Proteome Discoverer 1.4.1 against the Uniprot Mouse database from July 2014. Spectra generated were searched using carbamidomethylation on cysteine as a fixed modification, oxidation of methionine as a variable modification, 10 ppm parent tolerance and 1 Da fragment tolerance. All hits were required to be fully tryptic. The results were exported to Scaffold (version 4.3.2, Proteome Software Inc., Portland, OR) to validate MS/MS based peptide and protein identifications, and to visualize multiple datasets in a comprehensive manner. Confidence of protein identification in Scaffold is shown as \geq 95% confidence (green coloration) and 80% to 94% confidence (yellow coloration). Relative quantization of proteins was performed by summing all data from the 8 gel slices for a particular sample in Scaffold and then displaying the Quantitative Value from the program. This number gives an average total of non-grouped spectral counts for a protein divided by the total non-grouping spectral counts for the 8 mass spectral runs from the gels slices from each lane (http://www.proteomesoftware.com/). The Quantitative Value allows a relative quantitative comparison between a specific protein from different samples and relative abundance between proteins for a particular exudate sample.

Immunochemical detection of ECM proteins in wound exudates

For immunoblotting, 100 µg protein of each exudate sample were separated under reducing conditions on 4–15% Tris–HCl polyacrylamide gradient gels, and transferred to nitrocellulose membranes. Immunodetection was performed by incubating the membranes overnight at 4°C stirring with rabbit anti-type IV collagen polyclonal antibody at a dilution of 1:200 (Abcam ab19808), rabbit anti-nidogen 1 polyclonal antibody at a dilution of 1:500 (Abcam ab19808), rabbit anti-laminin polyclonal antibody at a dilution of 1:1,000 (Thermo PA1-32130), rabbit anti-type VI collagen polyclonal antibody at a dilution of 1:2,000 (Millipore AB7821), rabbit anti-type I collagen polyclonal antibody at a dilution of 1:2,000 (Millipore AB7821), rabbit anti-type I collagen polyclonal antibody at a dilution of 1:1,000 (Abcam ab21286), or rabbit anti-type I collagen polyclonal antibody at a dilution of 1:3,000 (Abcam ab21286), or rabbit anti-fibronectin polyclonal antibody at a dilution of 1:3,000 (Abcam ab21286), or rabbit anti-type I collagen antibody at a dilution of 1:3,000 (Abcam ab21286), or rabbit anti-fibronectin polyclonal antibody at a dilution of 1:5,000 (Jackson ImmunoResearch) and the chemiluminescent substrate Lumi-Light (Roche). Images were captured with the ChemiDoc XRS+ System (BioRad) and the analysis was performed with the ImageLah software (BioRad).

Quantification of proteolytic activity of wound exudates

Gelatinase assay. Proteolytic activity on fluorescent gelatin of wound exudates was assessed using a commercial kit (EnzCheck protocol Gelatinase/Collagenase Assay Kit, Molecular Probes, Life Technologies) in order to determine whether active SVMPs are present in the wound exudates. Exudate samples were collected as described above at 1 h, 6 h and 24 h after intramuscular injection of 100 μ g of *B. asper* venom. A venom dose of 100 μ g, instead of 50 μ g, was used in these experiments in order to increase the sensitivity of the assay for detection of proteinase activity. Exudate samples were pooled, centrifuged at 5,000 g for 3 min and kept at -70°C until the proteolytic assays were performed. 50 μ L of each exudate sample were incubated with 20 μ g of gelatin fluorescein conjugate substrate in a total volume of 200 μ L, in a 96 well microplate, protected from light, at room temperature, for 24 h. Each sample was tested in triplicate and a reagent blank was included. Fluorescence intensity was measured in the BioTek Synergy HT microplate reader setting the absorption filter at 495 nm and the emission filter at 515 nm. In order to determine whether proteolytic activity detected in exudate samples is due to SVMP or endogenous proteases, neutralization and zymography assays were performed with the exudate samples.

Neutralization assay. Exudate samples collected at 1 h were incubated with polyclonal antibodies against the SVMP BaPI for 20 min at 37°C prior to testing the exudate in the gelatinase activity assay described above. The antibody against BaP1 was obtained from the serum of rabbits immunized with BaP1; antibodies were purified by affinity chromatography. This antibody was used since BaP1 is the most abundant SVMP in the venom of adult *B. asper* snakes [18]. Previous studies showed that anti-BaP1 antibodies do not react with PIII SVMPs from *B. asper* venom and with MMPs [19,20]. Exudate samples collected at 1 h were selected for the neutralization assay since the highest proteolytic activity on fluorescent gelatin was observed at this time interval.

Zymography assay. Proteinase activity of exudate samples was visualized by gelatin zymography according to the method described by Herron et al. [21] and modified by Rucavado et al. [20]. Briefly, 10 µg protein of each exudate sample were separated on 7.5% SDS-polyacrylamide gel prepared with 0.50 mg/mL of Type A gelatin (Sigma Chemical Co., St Luis, MO). After electrophoretic run at 100 V, the gel was washed with 1% Triton X-100 for 30 min under agitation. Then, the gels were incubated with zymography buffer (50 mM Tris-HCl, 5 mM CaCl₂, 2 g/L NaN₃, pH 8.0) for 20 h at 37°C, stained for 2 h with Coomassie Blue R-250, and destained with water for 20 min.

Results

Pathological observations

The pathological alterations induced by *B. asper* venom were studied on mouse gastrocnemius muscle tissue over a time period of 24 h. Tissue sections from control mice injected with PBS showed normal histological features of skeletal muscle tissue with transverse bundles of muscle fibers, surrounded by connective tissue and normal vascular and nerve structures (Fig 1A). Tissue sections from mice injected with *B. asper* venom showed intense hemorrhage at 1 h (Fig 1B) and 6 h (Fig 1C), evidenced by the presence of abundant erythrocytes in the interstitial space surrounding muscle fibers. After 24 h of injection of *B. asper* venom, the hemorrhage decreased since the amounts of extravascular erythrocytes was reduced as compared to previous time intervals (Fig 1D), consistent with previous observations [7].

Moreover, tissue sections from mice injected with venom revealed prominent necrosis of skeletal muscle fibers at the first hour interval (Fig 1B and 1C). After 24 h following venom injection, the bundles of muscle fibers appeared partially lost and disorganized with a hyaline appearance (Fig 1D). These pathological observations also agree with previous studies [7,11,12]. Additionally, an infiltration of inflammatory cells was observed in tissue sections, especially after 6 h and 24 h of venom injection with a marked increment at 24 h (Fig 1D). The predominant cell type was polymorphonuclear leukocytes, although a proportion of mononuclear cells, i.e. macrophages, were also observed at 24 h. These observations also agree with previous studies [9].

On the other hand, CK activity of exudate samples collected after injection of venom was 228,776 \pm 47,137 IU/L at 1 h, 162,344 \pm 23,371 IU/L at 6 h, and 23,371 \pm 11,660 IU/L at 24 h (Fig 1E). CK is a marker of plasma membrane damage and cell death of skeletal muscle fibers; hence it appears that myotoxic activity of the venom is highest at one hour, decreasing afterwards. These results are in agreement with the muscle tissue damage observed in the histological analysis, which occurs early on in the course of envenoming.

Proteomic analysis of wound exudates

Wound exudate samples collected from mice injected with *B. asper* venom were decomplexed by SDS-PAGE for subsequent proteomic analysis. From the mass spectrometric analysis of the gel bands, a total of 537, 578, and 486 proteins were identified in exudates at 1 h, 6 h, and 24 h, respectively, with protein identification probability above 95% and minimum of two peptides (S1 Table). The most abundant proteins identified based on their Quantitative Value (see http://www.proteomesoftware.com/ for full description of term) were classified within the following groups: intracellular proteins (Table 1 and S2 Table), ECM proteins (Table 2), membrane proteins (S3 Table), coagulation factors (S4 Table), and proteinase inhibitors (S5 Table). Within each group, the proteins were organized by those that changed at least three fold as compared to another time and proteins which did not show significant change between the three time intervals, i.e. those whose amounts did not differ more than threefold between times. PLOS | NEGLECTED TROPICAL DISEASES

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Fig 1. Histological analysis and myotoxicity induced by *B. asper* venom in mouse gastrocnemius muscle. Groups of four mice were injected in the gastrocnemius with 50 µg of *B. asper* venom. After 1, 6 and 24 h of injection, mice were sacrificed and samples of exudate and muscle tissues were collected for quantification of creatine kinase (CK) activity and histological analysis, respectively. Tissue samples were collected at 1 h (B), 6 h (C) and 24 h (D) after injection and processed for embedding in parafilm. Tissue injected with PBS (A) was used as control. Notice abundant erythrocytes (arrow) at 1 h and 6 h, and antihiltration of inflammatory cells at 24 h (asterisk). Hematoxylin–eosin staining. Bar represents 100 µm. (E) CK activity of exudate was quantified using a commercial kit (see Methods for details). Results are expressed as mean ± S.D (n = 4).

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Table 1. More abundant intracellular proteins identified in wound exudates collected from mice at 1, 6 and 24 h after injection of B. asper venom, which changed at least three fold at one time as compared to another time.

Protein	Accession Number	Molecular mass	Quantitative Value		
			1h	6h	24h
Creatine kinase M-type	P07310	43 kDa	651	404	96
Fructose-bisphosphate aldolase	A6Z144	45 kDa	357	216	109
Phosphorylase	E9PUM3	88 kDa	300	294	47
Carboxylesterase 1C	P23953	61 kDa	200	168	0
Alpha-actinin-2	Q9JI91	104 kDa	190	298	59
Alpha-actinin-3	D88990	103 kDa	161	206	55
Actin, alpha skeletal muscle	P68134	42 kDa	120	258	220
L-lactate dehydrogenase	G5E8N5	40 kDa	105	87	34
Triosephosphate isomerase	P17751	32 kDa	98	96	27
Bisphosphoglycerate mutase	D70250	29 kDa	93	15	14
Colilin-1	P18760	19 kDa	83	10	92
Peroxiredoxin-5, mitochondrial	P99029 [2]	22 kDa	83	16	17
Glutathione peroxidase 1	P11352	22kDa	83	13	14
Isoform 2 of Myc box-dependent-interacting protein 1	D08539-2 [2]	48 kDa	83	22	0
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	Q8R429 [2]	109 kDa	80	77	0
UTP-glucose-1-phosphate uridylyltransferase	Q91ZJ5	57 kDa	74	78	11
Myosin-binding protein H	P70402	53 kDa	74	67	0
Ubiquitin-40S ribosomal protein S27a	P62983 [2]	18 kDa	74	15	26
Flavin reductase (NADPH)	Q923D2	22 kDa	74	15	22
L-lactate dehydrogenase B chain	P16125	37 kDa	65	18	80
Malate dehydrogenase, mitochondrial	P08249	36 kDa	65	63	14
Myosin-9	Q8VDD5	226 kDa	65	10	31
Phosphoglycerate kinase 1	P09411	45 kDa	58	47	15
Heat shock cognate 71 kDa protein	P63017 [4]	71 kDa	51	73	17
Aconitate hydratase, mitochondrial	Q99KI0	85 kDa	48	99	10
Thioredoxin	P10639	12 kDa	46	16	92
Isoform 3 of Elongation factor 1-delta	P57776-3	73 kDa	46	78	23
isoform Cytoplasmic of Fumarate hydratase, mitochondrial	P97807-2	50 kDa	46	15	69
Myosin-4	Q5SX39 [9]	223 kDa	43	620	529
isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q9D6R2	40 kDa	37	67	0
Glucose-6-phosphate isomerase	P06745	63 kDa	31	52	17
Ubiquitin-like protein ISG15	Q64339	18 kDa	28	90	34
3-ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	42 kDa	28	90	11
Protein disulfide-isomerase	P09103	57 kDa	28	17	69
Superoxide dismutase [Cu-Zn]	P08228	16 kDa	28	10	57
L-lactate dehydrogenase C chain	P00342	36 kDa	26	20	80
Peptidyl-prolyl cis-trans isomerase A	P17742	18 kDa	22	67	24
Adenylate kinase isoenzyme 1	Q9R0Y5 [2]	22 kDa	20	78	23
Myosin regulatory light chain 12B	Q3THE2	20 kDa	19	34	57
Isocitrate dehydrogenase [NADP], mitochondrial	P54071	51 kDa	19	78	0
Cofilin-2	P45591	19 kDa	17	78	46
Clathrin heavy chain 1	Q68FD5	192 kDa	17	18	92
Glutathione S-transferase P 1	P19157	24 kDa	16	15	80
Elongation factor 1-gamma	Q9D8N0	50 kDa	16	22	69
Carboxypeptidase N catalytic chain	09.11115	52 kDa	12	90	92

(Continued)

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Table 1. (Continued)

Accession Number	Molecular mass	Quantitative Value			
		1h	6h	24h	
Q61820 [2]	24 kDa	11	34	80	
P05977 [2]	21 kDa	10	26	59	
Q9CQA3	32 kDa	1	78	92	
P56480	56 kDa	1	34	80	
P58774	33 kDa	1	78	33	
	Accession Number Q61820 [2] P05977 [2] Q9CQA3 P56480 P58774	Accession Number Molecular mass Q61820 [2] 24 kDa P05977 [2] 21 kDa Q9CQA3 32 kDa P56480 56 kDa P58774 33 kDa	Accession Number Molecular mass Qua 1h 1h 1h Q61820 [2] 24 kDa 11 P05977 [2] 21 kDa 10 Q9CQA3 32 kDa 1 P56480 56 kDa 1 P58774 33 kDa 1	Accession Number Molecular mass Quantitative V 1h 6h Q61820 [2] 24 kDa 11 34 P05977 [2] 21 kDa 10 26 O9CQA3 32 kDa 1 78 P56480 56 kDa 1 34 P58774 33 kDa 1 78	

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A total of 222 intracellular proteins (Table 1 and S2 Table) and 13 membrane proteins (S3 Table) were detected in exudates, thus demonstrating direct or indirect cellular damage induced by the venom. The most abundant intracellular proteins detected in exudates were hemoglobin subunit beta-2 and creatine kinase M-type, in agreement with the hemorrhagic and inyotoxic activity of *B. asper* venom, respectively. Moreover, the creatine kinase M-type identified in the exudates was detected at the highest level at 1 h, and decreased over time until

Table 2, E	xtracellular matrix	proteins identified in wo	und exudates collected	from mice at 1, 6 a	and 24 h after injection of	B. asper venom.
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Protein	Accession Number	Molecular mass	Quantitative value		
			1 h	6 h	24 h
Proteins which changed at least three fold at one time as compared to	another time				
Basement membrane-specific heparan sulfate proteoglycan core protein	B1B0C7 [2]	469 kDa	83	56	0
Collagen alpha-1 (XVIII) chain	E9QPX1 [2]	182 kDa	74	22	0
Thrombospondin-1	P35441	130 kDa	65	16	11
Protein Col6a3	E9PWQ3	354 kDa	56	45	0
Collagen alpha-1 (XV) chain	A2AJY2 [2]	138 kDa	56	45	0
Collagen alpha-2(IV) chain	P08122	167 kDa	37	11	0
Collagen alpha-1(IV) chain	P02463	161 kDa	19	11	0
Nidogen-2	O88322	154 kDa	19	11	0
Nidogen-1	P10493	137 kDa	19	56	0
Collagen alpha-1(III) chain	P08121	139 kDa	12	45	23
Collagen alpha-2(I) chain	Q01149	130 kDa	16	22	57
Collagen alpha-1(I) chain	P11087	138 kDa	15	13	57
Laminin subunit gamma-1	P02468 [2]	177 kDa	1	56	46
Fibrillin-2	Q61555	314 kDa	1	22	0
Chondroitin sulfate proteoglycan 4	Q8VHY0	252 kDa	0	22	11
Fibrilin-1	A2AQ53 [2]	312 kDa	0	22	0
Collagen alpha-1(XII) chain	E9PX70 [3]	334 kDa	0	22	0
Proteins which did not change more than three-fold at any time as con	npared to another time				
Fibronectin	P11276	273 kDa	275	194	291
Thrombospondin-4	Q9Z1T2	106 kDa	65	34	46
Vitronectin	P29788	55 kDa	19	13	10
Dermatopontin	Q9QZZ6	24 kDa	19	11	23
Proteoglycan 4	E9QQ17 [4]	111 kDa	19	11	13
Collagen alpha-1(XIV) chain	B7ZNH7	193 kDa	12	16	29
Lumican	P51885	38 kDa	11	11	17

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reaching a six fold reduction at 24 h. These results are in agreement with the CK activity of exudates and the muscle tissue damage observed in the histological analysis. In contrast, there was a trend for cytoskeletal proteins, such as actin, myosin, and tropomyosin, to increase in the exudates over time, while most of cytosolic and mitochondrial proteins appeared at the first hour of venom injection, and decreased afterwards.

Of serum proteins, a total of 10 coagulation factors (S4_Table) and 14 proteinase inhibitors (S5_Table) were detected in exudates at various times. Fibrinogen beta and gamma chains appeared in the exudates at the first hour following venom injection and their abundance increased over time. Other coagulation factors detected whose amounts changed at least three fold as compared to values at other time were coagulation factor X, XII, and XIII. The inter alpha-trypsin inhibitor was the only proteinase inhibitor that increased at least threefold at 24 h as compared to 1 h and 6 h.

A total of 24 ECM proteins were identified in exudates, of which 21, 24, and 13 proteins were detected at 1 h, 6 h, and 24 h, respectively (Table 2). Most of these proteins showed a differential abundance greater than three-fold between samples collected at different times. The most abundant basement membrane (BM) protein detected in the wound exudates was BMspecific heparan sulfate proteoglycan core protein (perlecan), followed by alpha 1 and 2 chains of type IV collagen. Most of the BM components, such as heparan sulfate proteoglycan, type IV collagen and nidogen-2, appeared in the exudates at 1 h, and the amount decreased over time, largely becoming undetectable at 24 h. Conversely, the amount of laminin y-1 detected in the exudates increased at 6 h and 24 h, and the amount of nidogen-1 increased at 6 h as compared to 1 h and 24 h (Fig 2A). Other collagens, such as types VI, XV, and XVIII collagens, were present in the exudates at 1 h and 6 h, but were not detected at 24 h. Interestingly, type I collagen was also detected in the exudates at the first hour and its abundance increased at 24 h (Fig 2B). Other ECM proteins detected in the exudates whose abundance were greater at 6 h as compared to 1 h and 24 h were type III collagen, fibrillin 1 and 2, chondroitin sulfate proteoglycan 4, and type XII collagen. On the other hand, thrombospondin 1 appeared in the exudates at 1 h and decreased over time. Other ECM proteins detected in the exudates whose amounts did not vary more than threefold between times were fibronectin, thrombospondin-4, vitronectin, dermatopontin, proteoglycan 4, type XIV collagen, and lumican (Table 2).

Immunochemical detection of ECM proteins in wound exudates

Type IV collagen. Immunodetection of type IV collagen in wound exudates showed one prominent band of 107 kDa, with additional faint bands of 217 kDa, 172 kDa, 135 kDa and 70 kDa at 1 h and 6 h (Fig 3A). A reduction of intensity of the 107 kDa band was observed at 6 h as compared to 1 h. The molecular mass of the prominent band does not agree with the molecular mass of the alpha chains of type IV collagen (145–160 kDa). Thus, these bands very likely constitute degradation products. Type IV collagen and its degradation products were not detected in exudate samples collected at 24 h. Therefore, according to Western blot results, there is a rapid degradation of type IV collagen within the first 6 hours.

Laminin. Immunodetection of laminin showed the presence of this protein in the wound exudates at 1, 6, and 24 h with two prominent bands of 220 kDa and 140 kDa (Fig 3B). These bands agree with the molecular mass of some isoforms of alpha, beta, and gamma chains (130–200 kDa) of this protein. A reduction of intensity of the 220 kDa band was observed at 1 h as compared to 6 h and 24 h. The band of 140 kDa was not detected at 1 h. A reduction of intensity of the 220 kDa band were observed at 24 h as compared to 6 h.





Fig 2. Extracellular matrix proteins identified in wound exudates collected from mice after injection of *B. asper* venom. Groups of five mice were injected in the gastrocnemius with 50 µg of *B. asper* venom. After 1, 6 and 24 h of injection, mice were sacrificed and samples of exudate were collected, pooled and lyophilized. Proteomic analysis of exudates was performed as described in Methods. Proteins from the BM are included in (A), whereas other ECM proteins are depicted in (B). Only proteins whose amount varied at least three fold between time intervals were included in this figure. Notice that the level of BM proteins and non-fibrillar collagens are higher at 1 h, whereas type I collagen levels are higher at 24 h.

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Nidogen 1. Western blot analysis of wound exudates for nidogen 1 showed two prominent bands of 120 and 40 kDa (Fig 3C), which are likely to be degradation fragments. A reduction of the 40 kDa band with an increase of the 120 kDa band was observed over time.

Type I collagen. Immunodetection of type I collagen showed three bands corresponding to proteins of 135 kDa, 120 kDa and 107 kDa at 1 h and 6 h (Fig 3D). An increase in the intensity of these bands, in particular the 120 kDa, was observed at 6 h as compared to 1 h. These bands agree with the molecular mass of some isoforms of alpha 1 (120 kDa) and alpha 2 (115 kDa) chains. Collagen I and degradation products were not detected at 24 h in the exudates.

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Fig 3. Western blot analysis of extracellular matrix components in wound exudates collected from mice after injection of *B. asper* venom. Groups of five mice were injected in the gastrocnemius with 50 µg of *B. asper* venom. After 1, 6 and 24 h of injection mice were sacrificed and samples of exudates were collected, pooled and lyophilized. Afterwards, 100 µg of protein of each sample were separated under reducing conditions on 4–15% Tris–HCI SDS-PAGE, and transferred to nitrocellulose membranes. Immunodetection was performed with (A) anti-type IV collagen (CoI IV), (B) anti-taminin, (C) antinidogen 1, (D) anti-type I collagen (CoI I), (E) anti-type VI collagen (CoI VI), and (F) anti-fibronectin. The reaction was detected using an anti-rabbit peroxidase antibody and a chemiluminescent substrate. Images were obtained with the ChemiDoc XRS+ System (BioRad).

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Overall there is a trend of increasing abundance of type I collagen in the exudates by Western blot analyses at 6 h as compared to 1 h.

Type VI collagen. Immunoblotting of wound exudates for type VI collagen showed one prominent band of 230 kDa, and a faint band of 118 kDa at 1 h and 6 h (Fig 3E). The presence of a band of 118 kDa in the first 6 h indicates degradation of type VI collagen early in the course of envenoming. A reduction of the 118 kDa band, with an increase of the 230 kDa band, was observed over time. Therefore, according to Western blot results, type VI collagen is degraded in the first hours, and then type VI collagen increases and appears in the exudates collected at 24 h.

Fibronectin. Immunodetection of fibronectin showed two bands of 265 kDa and 236 kDa, with additional faint bands of 175 kDa, 140 kDa, and 100 kDa at 1 h, 6 h and 24 h (Fig 3F). An increase of intensity of the most abundant bands and a reduction of the faint bands were observed at 24 h as compared to 1 h and 6 h. The molecular mass of the 265 kDa band agrees

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Fig 4. Proteolytic activity of wound exudates collected from mice after injection of *B. asper* venom. Groups of five mice wera injected in the gastrocnemius with 100 µg of *B. asper* venom. After 1, 6 and 24 h, mice were sacrificed and samples of exudates were collected. (A) Proteolytic activity of exudate samples was measured after 24 h of incubation with gelatin fluorescein conjugate using a commercial kit (EnzCheck protocol Gelatinase/ Collagenase Assay Kit, Molecular Probes, Life Technologies) as described in Methods. Results are expressed as mean ± S.D (n = 3) of Fluorescence Arbitrary Units (A.U.). (B) Exudate samples were separated in a 7.5% SDS-polyacrylamide gel containing 0.50 mg/mL of Type A gelatin. The gel was incubated at 37°C and then stained with Coomassie blue R-250. MMP: matrix metalloproteinase (see text for explanation); M: lane corresponding to molecular mass markers.

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with the molecular mass expected (262 kDa). Therefore, according to Western blot results, fibronectin is degraded in the first hours, but it remains present at 24 h in the exudates.

Proteolytic activity of wound exudates

In order to determinate whether SVMP or endogenous proteases are active in the wound exudates, proteolytic activity assays of exudate samples were performed. Exudate samples collected from mice injected with *B. asper* venom at 1 h showed the highest proteolytic activity on gelatin fluorescein conjugate compared with samples collected at 6 h and 24 h. (Fig 4A). When exudates collected at 1 h were incubated with polyclonal antibody against the SVMP BaP1, the proteolytic activity of the exudates was almost completely inhibited since only 8% of the activity remained.

Using zymography several gelatinolytic bands were detected corresponding to proteins of 50–150 kDa in the exudate samples collected at different times (Fig 4B). An increase of two main bands of about 100 kDa and 60 kDa was observed in exudates collected at 6 h and 24 h. These molecular masses are consistent with the latent forms of matrix metalloproteinases (MMP) 9 and 2, respectively [22]. Therefore, the zymography showed an increase of proteolytic activity of endogenous MMPs in exudate over time. Furthermore, bands corresponding to the molecular mass of the SVMP BaP1 were not detected in the zymographic analysis of exudate samples.

Discussion

Envenoming by venomous snakes gives rise to a complex pathophysiology by virtue of the complexity of the venoms and the fact that the toxins in the venom produce manifold effects in the tissues. Proteomic analysis of wound exudates collected in the vicinity of affected tissue constitutes a powerful approach to study the pathogenesis of tissue damage induced by snake venoms from a more comprehensive perspective [13–17], thus complementing histological, ultrastructural and biochemical analyses. This methodological tool has been used to investigate the early alterations provoked by *B. asper* venom [15] and some of its toxins, especially myotoxic PLA₂s and hemorrhagic SVMPs [13,14,16], as well as the inhibitory effects of antivenoms and low molecular mass inhibitors [15]. However, analyses in these previous studies were performed at a single time interval after injection, thus precluding the understanding of these events from a time-course perspective. In this study, we investigated the dynamics of local effects induced by *B. asper* venom in the gastrocnemius muscle of mice at various time intervals.

Our histological and biochemical observations agree with previous studies showing a rapid development of myonecrosis and hemorrhage, followed by an inflammatory process characterized by the infiltration of neutrophils and macrophages at later time intervals [4,5,7,9,10]. Previous works on venom-induced myonecrosis have quantified CK activity in plasma [6], where the highest levels were observed at 3 h post envenoming. In contrast, in exudates, highest CK levels are higher than in plasma, and peak activity occurs at 1 h instead of 3 h. This difference may be attributed to the kinetics of absorption of this enzyme into the circulation after its release from damaged muscle fibers, since exudate was collected close to the venom-injected muscle. In agreement with previous pathological and proteomic studies, intracellular proteins were abundant in exudates, as a consequence of the cytotoxic effect of venom, especially on skeletal muscle fibers [7,11,14,15]. The time-course analysis of the intracellular proteins in exudate underscores that most of the cytosolic and mitochondrial proteins appear early on due to the rapid action of myotoxic PLA₂s and PLA₂ homologues in muscle tissue [7,11,14,15,23], followed by a decrease of these proteins. Most of these proteins are derived from the cytosol of skeletal muscle fibers since myotoxic PLA2s induce a rapid disruption of the integrity of muscle cell plasma membrane [4,7,24]. The high CK activity of exudates at 1 h and our histological observations corroborate the early onset of myonecrosis in the course of envenoming and agree with proteomic analysis.

In contrast to cytosolic proteins, most of the cytoskeletal proteins, such as actin, myosin, and tropomyosin, are more abundant in exudates collected at later time periods. This late increment suggests that the presence of cytoskeletal protein fragments in the exudate depends on the action of proteinases that release these structural components from damaged cells. A prominent calcium influx in muscle cells occurs after venom-induced plasma membrane damage [25,26]. An increased calcium concentration in the cytosol results in the activation of calpains, which might hydrolyze cytoskeletal components [27]. Subsequently, proteinases derived from inflammatory cells arriving at the necrotic tissue may also contribute to proteolysis of muscle cytoskeletal proteins [9,10]. Thus, the proteomic analyses reveals two 'waves' of release of intracellular proteins to exudates: an early release of cytosolic and mitochondrial proteins, which depends on the rapid myotoxin-induced membrane damage, and a more delayed release of cytoskeletal protein fragments, which is due to proteolytic degradation.

The presence of cell membrane-associated proteins may be evidence of direct or indirect cellular damage induced by the venom. Moreover, proteolysis of these components, either by venom or endogenous proteinases, may cause their 'shedding' and diffusion to the exudate compartment. It is tempting to speculate that, in addition to being a passive reflection of venom-induced plasma membrane damage, the release of these protein fragments may also play a functional role in cellular signaling associated with inflammatory and reparative events. However, the pathological relevance of the hydrolysis of these proteins in the overall mechanism of local tissue damage induced by snake venoms has not been established and needs further study.

The presence of ECM proteins in wound exudates reflects the cleavage by either venomderived proteinases or endogenous proteinases, such as MMPs, generated in the course of the inflammatory response. The degradation of ECM is a relevant component of viperid venom-

induced tissue damage, and proteomic analysis has been particularly useful in revealing a complex pattern of hydrolysis [14-16]. Previous studies detected B. asper venom components in muscle homogenates of mice during the first week after experimental envenoming [28]; however, the activity of these toxins has not been previously addressed. When assessing the proteinase activity of exudates, highest activity was detected in samples collected after 1 h of envenoming; here we demonstrate that this is mainly due to the action of SVMPs, since antibodies against BaP1, the most abundant proteinase in B. asper venom [29], almost fully inhibited exudate-induced proteolysis. However, this enzymatic activity decreased over time, probably as a consequence of diffusion of venom components from the injected muscle or of inhibition by plasma or tissue-derived proteinase inhibitors. It is likely that activity at later time intervals, i.e. 24 h, is mostly due to endogenous MMPs generated in the course of the inflammatory response, such as MMP-9 and MMP-2, which was confirmed by the detection with zymography of the wound exudates, although it remains possible that some venom proteinases persisting in the tissue may also contribute to this observation. These results agree with previous studies which demonstrated an increase in the expression of MMP-9 and MMP-2 in muscle tissue injected with B. asper venom [28] or with purified SVMP and PLA2 toxins [30]. Taken together these findings suggest that the hydrolysis of ECM is mainly due to SVMPs in the early stages of envenoming, while endogenous MMPs participate later in the course of envenoming.

A large body of experimental evidence indicates that BM and related ECM components that provide stability to microvessel structure are the key targets of hemorrhagic SVMP [5,13,14,16,31-34]. Moreover, SVMP-induced hemorrhage occurs very fast after injection [12,35-37]. Therefore, the presence of ECM components in wound exudates during first hour as compared to later time periods may offer important insights for understanding the mechanism of action of hemorrhagic SVMPs. Regarding BM components, the presence of degradation products of perlecan, type IV collagen, nidogen, and laminin in wound exudates underscores a rapid and drastic damage of BM structure. In particular, perlecan and type IV collagen are abundant in exudates after the first hour, when hemorrhagic events have occurred, and then their amounts decrease over time, as they were not detected by proteomic analysis at 24 h. Western blot analysis of exudate confirmed the presence of fragments of type IV collagen 1 h after venom injection.

Perlecan is the most abundant BM protein detected in the wound exudates during the first hour. In previous proteomic studies, we have found that the relative amount of perlecan in wound exudates induced by a hemorrhagic SVMP was greater as compared to a non-hemorrhagic one [13], but similar when compared to other hemorrhagic SVMPs [16], and its presence was abolished when *B. asper* venom was previously incubated with batimastat [15], a metalloproteinase inhibitor. Such findings, together with our data, suggest that degradation of perlecan in early stages of envenoming may play an important role in the hemorrhagic mechanism of SVMPs. This proposal agrees with the known structural role of perlecan in BM [38–42]. In addition, mutations in the perlecan gene in mice have been associated with loss of BM integrity in different tissues [43–45], including microvasculature of brain and skin, which cause severe bleedings due to dilatation and rupture of microvessels [45].

On the other hand, previous proteomic studies using similar models have not detected type IV collagen in wound exudates induced by *B. asper* venom or its toxins [13–16]. However, several studies using *in vitro* and *in vivo* models have identified type IV collagen as one of the most likely key components degraded by hemorrhagic SVMPs and associated with the initial microvessel destabilization and hemorrhage [5,13,16,34,46,47]. Our present proteomic results did identify fragments of type IV collagen in exudates at 1 h, thus agreeing with previous immunohistochemical and immunochemical evidence [13,16]. In addition, according to Western blot analysis, degradation products of type IV collagen appear in exudates in samples collected at 1 h. Such early appearance of type IV collagen and perlecan strongly suggest that their degradation is due to the direct proteolytic activity of SVMPs. The hypothesis that type IV collagen is a key target in the hemorrhagic mechanism of SVMPs is compatible with the structural role of this collagen in the mechanical stability of BM, as it is stabilized by covalent cross-links [41,42,48–51]. In addition, mutations on type IV collagen genes have been associated with pathological alterations in microvessels and with hemorrhage in brain, kidney and lungs in mice and humans [52–58]. Thus, the rapid hydrolysis of perlecan and type IV collagen after injection of *B. asper* venom supports the view that BM destabilization leading to hemorrhage is likely to depend on the degradation of these mechanically-relevant components.

Nidogen 2 appeared in early time periods in wound exudates, in agreement with previous proteomic studies [13,15], and then it decreased over time in our proteomics analysis. Since nidogen 2 is more abundant in the BM of blood vessels [59], and its time-course dynamics of appearance in exudates is similar to that observed for type IV collagen and perlecan, the release of nidogen 2 might be associated with vascular BM damage. In contrast, taken together the proteomic and Western blot analyses showed that nidogen 1 increased over time in wound exudates. In addition, nidogen 1 and 2 have been detected in plasma of healthy mice [60], which could explain the presence of nidogen 1 in the wound exudates according to Western blot results. On the other hand, laminin γ 1, which is widely distributed [61,62], also increased over time in wound exudates. The time-course variation of the molecular masses of immunoreactive bands in the cases of nidogen 1 and laminin underscores the dynamics of degradation of these components over time. Furthermore, Escalante et al. [13] demonstrated similar patterns of degradation for nidogen and laminin in muscle tissue induced by hemorrhagic and nonhemorrhagic SVMPs.

Our observations allowed the analysis of the time-course dynamics of the hydrolysis of nonfibrillar collagens associated with the BM, such as types VI, XV, and XVIII collagens. As in the case of type IV collagen and perlecan, hydrolysis of these components was highest at 1 h, hence indicating a rapid degradation, probably by venom proteinases. These collagens connect the BM with fibrillar collagens of the matrix [39,63], and are known to play a relevant role in the mechanical stability and integration of BM with connective tissue [39,63]. Hence, the hydrolysis of these components by SVMP might be also critical for capillary wall destabilization, as have been previously proposed [13,64]. Alternatively, the increase of these collagens in exudates might be consequence of BM damage after the hydrolysis of other components, such as type IV collagen and perlecan. Type VI collagen is more abundant in the BM of muscle cells [65–67]; thus the increment of non-degraded type VI collagen chains in exudates could reflect synthesis *de novo* during reparative and regenerative events in muscle tissue. The role of the degradation of these collagens in the initial destabilization of BM induced by hemorrhagic SVMP is an issue that should be further investigated.

Other ECM components of interest detected in the proteomic analysis are collagen I and fibronectin. Collagen I is a fibril-forming collagen distributed in non-cartilaginous connective tissues such as skin and connective tissue of muscle [63]. According to proteomic results, the relative abundance of collagen I in exudates is higher at 24 h as compared to 1 and 6 h. This late hydrolysis of collagen I could be result of the action of endogenous MMPs synthesized during the course of inflammation in the damage tissue. Fibronectin was detected in the exudates both in proteomic and immunochemical analyses. This protein can be found in two forms: plasma fibronectin, which is a soluble molecule synthesized by hepatocytes, and cellular fibronectin, which is produced in the tissues and is incorporated in the ECM [62]. Thus, the presence of fibronectin in exudates could be either a consequence of plasma exudation or

hydrolysis from the ECM. According to proteomic analysis, the amount of fibronectin in exudates does not change over time; however, on the basis of Western blot analysis, it appears to be more degraded at early time periods most likely due to the action of SVMPs.

Taken together, our observations highlight a dual pattern of ECM protein degradation and appearance in exudates. Types IV and VI collagens, perlecan, nidogen and fibronectin show a higher degradation early on in the course of envenoming, correlating with the rapid action of SVMPs upon venom injection, as demonstrated by the proteinase activity of exudates. The rapid action of SVMPs on various key components of the BM is likely to be causally related to microvessel damage and hemorrhage. In the case of the fibrillar collagen I, it seems to be degraded predominantly by endogenous MMPs at later time periods, during the inflammatory reaction that ensues in the tissue as a consequence of venom-induced damage, as evidenced by zymography.

The presence of abundant plasma proteins in the exudate, as revealed hy proteomic analysis, is a consequence of plasma exudation as a result of edema and increment in vascular permeability induced by the venom [68,69]. Some of the plasma proteins detected are acute-phase proteins, proteinase inhibitors and coagulation factors. Of interest is the increase of fibrinogen and the inter α-trypsin inhibitor heavy chains over time.

The presence of fibrinogen in exudates might be secondary to the inflammatory exudation induced by the venom since this protein is typically found in plasma at high concentrations [70]. Previous proteomic studies have found fibrinogen in the wound exudates induced by *B. asper* venom and its toxins, especially SVMP BaP1, early in the course of envenoming [14,15]. Our data show an increase of fibrinogen in wound exudates over time. This increment might be consequence of fibrin clot formation in capillary walls, due to vascular damage induced by SVMPs [12,14,35], and also to fibrin formation in the extravascular interstitial space, followed by fibrinolysis by endogenous proteinases [70], thus explaining their higher amounts in exudates collected at later time intervals.

The inter- α -trypsin inhibitor heavy chains are mainly secreted into the blood by the liver as serum protease inhibitor whose concentration increases in inflammatory conditions [71]. The effect of these proteins in tissues has been associated with both inflammatory and anti-inflammatory activities [71–73]. Moreover, these proteins can be covalently linked to hyaluronan, exerting functions on cell migration and ECM remodeling under physiological and pathological conditions [74,75]. Thus, the increase of inter- α -trypsin inhibitor heavy chains detected in exudates might be due to an acute-phase inflammatory response and to the tissue inflammation as a consequence of venom-induced damage.

In conclusion, the proteomic analysis of wound exudates performed in this study provides a more complete understanding of the time-course dynamics of muscle tissue damage induced by *B. asper* venom. These observations, together with Western blot and histology data, provide a more integrated view of venom-induced local tissue damage (Fig 5). The early presence of cytosolic and mitochondrial proteins in exudates, as compared to the later increase of cytoskel-etal proteins, confirms the rapid cytotoxic effect of venom, followed by the action of endoge-nous proteinases in the cytoskeleton of damaged muscle fibers. On the other hand, the early presence of BM and other BM-associated ECM components in exudates, together with venom-derived proteolytic activity of exudates, strongly suggest the hydrolysis of these components by SVMPs in the early stages of envenoming. In contrast, the increment of some ECM proteins in the intervals is likely to be due to the action of endogenous MMPs or to their synthesis *de novo* during tissue remodeling associated with inflammation and reparative processes. Finally, the time-course of appearance in wound exudates of type IV collagen and perlecan supports the role of the hydrolysis of these BM components in the mechanism of microvascular damage induced by hemorrhagic SVMP.

Proteomic Analysis of Venom-Induced Tissue Damage





Fig 5. Early and late pathological events induced by the venom of *Bothrops asper* in muscle tissue. (A) The venom of *B*, asper induces a rapid cytotoxic (especially myotoxic) and hemorrhagic effects evidenced by the early release of cytosolic and mitochondrial proteins, and the degradation of BM and related ECM, respectively. (B) Then, MMPs and other endogenous proteinases are associated with tissue remodeling and degradation of cytoskeletal proteins, especially in skeletal muscle, as part of the inflammatory reaction later on in the course of envenoming. BM: basement membrane; EC: endothelial cells; ECM: extracellular matrix; SVMPs: snake venom metalloproteinases; SV PLA₂s: snake venom phospholipases A₂; EP: endogenous proteases.

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Supporting Information

S1 Table. List of all proteins identified in wound exudates collected from mice at 1, 6 and 24 h after injection of *B. asper* venom.

(PDF)

S2 Table. Intracellular proteins identified in wound exudates collected from mice at 1, 6 and 24 h after injection of *B. asper* venom.

(PDF)

S3 Table. Membrane proteins identified in wound exudates collected from mice at 1, 6 and 24 h after injection of *B. asper* venom. (PDF)

S4 Table. Coagulation factors identified in wound exudates collected from mice at 1, 6 and 24 h after injection of *B. asper* venom.

(PDF)

S5 Table. Serum proteinase inhibitors identified in wound exudates collected from mice at 1, 6 and 24 h after injection of *B. asper* venom. (PDF)

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Author Contributions

Conceived and designed the experiments: CH TE AR JMG JWF. Performed the experiments: CH JKAM AF TE AR JMG JWF. Analyzed the data: CH JKAM AF TE AR JMG JWF. Contributed reagents/materials/analysis tools: CH TE AR JMG JWF. Wrote the paper: CH TE AR JMG JWF.

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ARTICULO IV



Review

A Comprehensive View of the Structural and Functional Alterations of Extracellular Matrix by Snake Venom Metalloproteinases (SVMPs): Novel Perspectives on the Pathophysiology of Envenoming

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Abstract: Snake venom metalloproteinases (SVMPs) affect the extracellular matrix (ECM) in multiple and complex ways. Previously, the combination of various methodological platforms, including electron microscopy, histochemistry, immunohistochemistry, and Western blot, has allowed a partial understanding of such complex pathology. In recent years, the proteomics analysis of exudates collected in the vicinity of tissues affected by SVMPs has provided novel and exciting information on SVMP-induced ECM alterations. The presence of fragments of an array of ECM proteins, including those of the basement membrane, has revealed a complex pathological scenario caused by the direct action of SVMPs. In addition, the time-course analysis of these changes has underscored that degradation of some fibrillar collagens is likely to depend on the action of endogenous proteinases, such as matrix metalloproteinases (MMPs), synthesized as a consequence of the inflaminatory process. The action of SVMPs on the ECM also results in the release of ECM-derived biologically-active peptides that exert diverse actions in the tissue, some of which might be associated with reparative events or with further tissue damage. The study of the effects of SVMP on the ECM is an open field of research which may bring a renewed understanding of snake venom-induced pathology.

Keywords: proteomics; exudate; extracellular matrix; basement membrane; hemorrhage; snake venom metalloproteinases; FACITs

1. Extracellular Matrix Pathology: An Elusive Aspect in the Understanding of Snakebite Envenoming

Snakebite envenoming is a public health problem of high impact on a global basis, especially in tropical and subtropical regions of Africa, Asia, Latin America, and parts of Oceania, causing morbidity, mortality, and a wave of social suffering [1–4]. The spectrum of pathological and pathophysiological effects inflicted by snake venoms is very wide, and encompasses both local tissue damage and systemic, life-threatening alterations [5]. Venoms of snakes of the family Viperidae are rich in hydrolytic enzymes, having a high content of zinc-dependent metalloproteinases (SVMPs), phospholipases A₂ (PLA₂s), and serine proteinases (SVSPs), although the relative proportions of these enzymes varies among venoms [6,7]. SVMPs are known to play multiple roles in the local and systemic effects



induced by viperid venoms in natural prey and humans [8–10]. SVMPs induce local and systemic hemorrhage, myonecrosis, blistering, dermonecrosis, edema, and coagulopathies, in addition to being algogenic and strongly pro-inflammatory [9–12]. Furthermore, the pathological alterations induced by SVMPs in skeletal muscle tissue contribute to the poor muscle regeneration characteristic of these envenomings [13,14].

Several aspects of the local pathological effects induced by SVMPs have been studied, such as the microvascular damage leading to hemorrhage [15,16], skeletal muscle necrosis and poor muscle regeneration [13,14,17], blistering, and dermonecrosis [18,19], as well as the identification of inflammatory mediators responsible for pain, edema, and leukocyte infiltration [20–22]. To a great extent, these alterations are considered to be associated in some manner with the action of SVMPs on the extracellular matrix (ECM). However, the specific effects induced by SVMPs as a result of their action on ECM have been investigated only to a limited extent, being mostly focused on the structural damage to basement membrane (BM) components of capillary blood vessels [15,16,23,24]. The reasons behind the paucity of information in this aspect of envenoming have to do mostly with methodological limitations and to the complexity of ECM structure and function. In his celebrated book The Logic of Life, François Jacob stated "The alternative approach to the history of Biology involves the attempt to discover how objects become accessible to investigation thus permitting new fields of science to be developed" [25]. It is then relevant to discuss how the alterations in ECM by snake venoms and SVMPs have become accessible to investigation.

The ability of SVMPs to degrade diverse ECM proteins has been assessed in vitro, mostly through SDS-PAGE, by observing the degradation patterns of ECM components incubated for various time intervals with the enzymes (Figure 1A). This has led to a wealth of information showing that SVMPs have a relatively wide spectrum of activity over substrates such as laminin, nidogen/entactin, type IV collagen, and fibronectin [23,26–33]. Likewise, SVMPs have been shown to hydrolyze proteoglycans in vitro, such as heparan sulphate proteoglycan and aggrecan [15,34]. However, this experimental approach has limitations, as hydrolysis has been studied in isolated ECM components and, therefore, the experimental conditions do not reproduce the complex landscape of these proteins in the tissues, which may determine their susceptibility to these enzymes. The ability of snake venoms to degrade hyaluronic acid, a glycosaminoglycan of the ECM, by the action of hyaluronidases, has been assessed using various in vitro methods [35].

Studies using classical ultrastructural methods, i.e., transmission electron microscopy (TEM), have focused on the alterations in BM structure by SVMPs (Figure 1B), as well as on the disorganization of fibrillar collagen bundles [36,37]. This approach, nevertheless, is not able to detect alterations in components of the ECM that are not observed at the ultrastructural level. Moreover, tissue processing for TEM reduces the actual thickness of BM, as demonstrated by atomic force microscopy [38]. Histochemistry and immunohistochemistry techniques, on the other hand, provide a more specific assessment of ECM components (Figure 1C,D), but the number of studies with SVMPs and hyaluronidases is limited (examples are [14,16,31,39,40]). Moreover, these procedures allow the detection of specific components, but do not provide a broad view of ECM alterations. More recently, the application of Western blot techniques to study the degradation of ECM components in vivo by SVMPs has provided novel clues to understand these phenomena, in particular regarding hydrolysis of BM components [15,24,41] (Figure 1E). Nevertheless, this method has the limitation that only the proteins to which antibodies are directed can be detected, thus precluding a comprehensive analysis of ECM alterations. Overall, the methodologies described have provided valuable, albeit limited, information of the action of SVMPs on the ECM.

To this end, the introduction of proteomic analysis to the field of pathology has represented a significant step forward in the study of disease at clinical and experimental levels, and in the search of biomarkers (Figure 1F) (see for example [42–47]). This methodological platform offers complementary and often advantageous outcomes as compared to other methods mentioned above. Of particular relevance is that it is not focused on the detection of particular tissue components, as occurs with the immunological-based methods, but instead provides unbiased information on many tissue components at a time. This approach thereby opens a greater aperture through which overt and subtle tissue alterations can be detected. In 2009, our group first utilized a proteomics-based approached to study the tissue damage induced by snake venoms and by specific venom components, such as SVMPs and myotoxic PLA₂s [40]. This initial watershed contribution demonstrated the great potential of this methodology to understand the pathological effects of snake venoms and toxins, and was followed by a series of studies on this topic [15,24,40,41,48,49]. The present review summarizes the key findings that have emerged from these investigations in relation to the alterations induced by SVMPs in the ECM and how these inform our understanding of the role of SVMPs and envenoming.



Figure 1. Experimental approaches to study the action of snake venom metalloproteinases (SVMPs) on the extracellular matrix (ECM). (A) In vitro analysis of hydrolysis of ECM proteins by SVMPs. Basement membrane (BM) preparations, such as Matrigel in this figure, or isolated ECM proteins, are incubated with SVMPs and the mixture is then analyzed by SDS-PAGE to assess the cleavage products; C: control Matrigel; degradation induced by PI and PIII SVMPs is shown. Molecular mass markers are shown to the left (reproduced from [31], copyright 2006 Elsevier); (B) Transmission electron microscopy assessment of ECM damage by analyzing the alterations in tissues from animals injected with SVMPs. A disrupted capillary vessel with damage to BM is shown after the injection of a hemorrhagic SVMP; 10,000 × (reproduced [50], copyright 2006 Elsevier); (C) Histochemical assessment of collagen degradation. A histology section of muscle tissue stained with Sirius Red, which stains collagen, and Fast Green, which stains proteins, is shown; 200 × (reproduced from [14], copyright 2011 PLOS); (D) Immunohistochemistry staining of a sample of skin injected with a SVMP. The blue staining corresponds to Hoechst 33258, which stains nuclei, whereas the red staining corresponds to immunostaining with a monoclonal antibody against type IV collagen; 400 ×; (E) Western blotting analysis of type VI collagen in samples of exudates collected from tissue injected with PI, PII and PIII SVMPs. Different patterns of hydrolyzed fragments are observed. Molecular mass markers are depicted to the left (reproduced from [24], copyright 2015 PLOS); (F) Mass spectrometry analysis of proteins in exudates collected in the vicinity of tissue injected with SVMPs allows the identification of degradation products of many types of ECM proteins. A mass spectrum is shown for illustrative purposes.

2. Methodological Aspects of Proteomics Studies

Proteomic analysis of tissue samples in pathological settings can be performed by studying tissue homogenates. This approach has been followed in the analysis of alterations induced by SVMPs of the venom of Bothrops jararaca in the skin [51]. One problem for analyzing proteomics of ECM in tissue homogenates is that extraction of ECM proteins is difficult and, therefore, the "matrisome", i.e., the ECM proteome, is often underrepresented in tissue homogenate samples [47]. As with most experimental approaches to identify markers of particular biological or pathological processes, proteomic assessment of compartments nearest to the site of interest is likely to give best results. Thus, our group has developed a strategy based on the proteomic analysis of exudates collected in the vicinity of tissues injected with snake venoms or isolated toxins, such as SVMPs. In these studies we employed a mouse model extensively used for the investigation of histological and ultrastructural alterations after injection of venoms or purified toxins. Specifically we inject SVMPs intramuscularly in the gastrocnemius muscle of mice and then, at various time intervals, animals are sacrificed and an incision made in the skin overlying the affected muscle. A heparinized glass capillary vessel is then introduced under the skin, and the exudate fluid is collected by capillarity (Figure 2). In this experimental setting, the effect of SVMP inhibitors or of antivenom antibodies can be assessed either by preincubating SVMPs with inhibitors/antibodies or by injecting these molecules after envenoming [48,49]. In parallel, the affected muscle tissue can be collected and either fixed and processed for histological, ultrastructural or immunohistochemical observation, or homogenized for immunological analyses, i.e., Western blots or ELISA. One limitation of this approach is the generation of appropriate controls. Unfortunately, exudates cannot be collected from control animals, i.e., mice injected with saline solution, because edema and exudate do not develop in these conditions. Therefore, these studies have to be performed using other types of controls, such as other toxins, and then comparing the differences in the outcomes of proteomics analysis between different treatments.

Once exudate samples are collected, they are rapidly freeze-dried in order to ensure the stability of the sample. Aliquots of exudates are separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Then, the gel lanes corresponding to each sample are cut into ten equal size slices, corresponding to regions of varying ranges of molecular masses. After reduction and alkylation, gel slices are submitted to trypsinization, and tryptic peptides are analyzed by LC/MS/MS mass spectrometry analysis. Lists of peaks are generated from the raw data against the Uniprot Mouse database. The results from the searches are exported to Scaffold (version 4.3.2, Proteome Software Inc., Portland, OR, USA). Scaffold is used to validate MS/MS based peptide and protein identifications, and also to visualize multiple datasets in a comprehensive manner. Relative quantification of proteins is accomplished by combining all data from the 10 gel slices for a particular sample in Scaffold and then displaying the Quantitative Value from the program. This format of presentation allows for a comparison of the relative abundance of a specific protein presenting different samples. A detailed account on the methodology used in these studies can be found in Escalante et al. [40] (Figure 2).

The separation of protein bands in the gels into ten slices allows the determination of whether proteins in the samples are degraded or not. The amount of a given protein in a particular gel slice is determined as the percentage of that protein in all slices. Knowing the molecular mass of the native protein, the percentage of the protein migrating in regions of molecular mass lower than its native mass corresponds to the percentage of degradation of that protein in the sample [40]. The presence of a protein, or a protein fragment, in an exudate is likely to be due to one of the following reasons: (a) The protein has been degraded by proteinases present in the venom; (b) the protein has been degraded by proteinases derived from the inflammatory reaction to envenoming; (c) the protein has been released, without degradation, from a storage site in the ECM; (d) the protein has been synthesized during the process of envenoming and the ensuing inflammatory reaction; (e) the protein is present in the blood plasma and reaches the exudate as a consequence of the increment in vascular permeability; and (f) the protein has been released from cells due to the cytotoxic action of venom components (Figure 3). The first two possibilities can be detected by demonstrating the

presence of fragments of the proteins in regions in the gel corresponding to molecular masses lower than those of the native proteins. Noteworthy, in addition to ECM-derived protein fragments, exudates collected from the site of SVMP injection may also include plasma proteins, intracellular proteins, and proteins of membrane origin, among others.



Peptides identification by MS

Figure 2. Basic experimental protocol for the proteomics analysis of exudates collected from tissues injected with SVMPs. Mice are injected intraunuscularly in the gastrocnemius with SVMPs, or with mixtures of SVMPs and antibodies or inhibitors. At various time intervals animals are sacrificed and a sample of exudate is collected with a heparinized capillary vessel after sectioning the skin underlying the affected region. Upon separation of exudate proteins on SDS-PAGE and staining, sections of the gel are cut, reduced, carboxymethylated, and trypsin-digested, and then submitted to proteomic analysis (see text for more details). The identity of ECM proteins in the exudate and the extent of degradation are then assessed. Magnification of the histology section: 200 ×.

Table 1 shows the most abundant ECM proteins that have been detected in the proteomics analysis of exudates collected from tissues injected with SVMPs.

Proteomics analyses need to be validated by complementary experimental approaches. In our studies, Western blot analysis of proteins of particular interest has been utilized for validation. These analyses have been performed either in the same exudate samples on which proteomic analyses were performed or in homogenates of tissues injected with the SVMPs, such as skeletal muscle or skin [15,24]. Another complementary approach is the use of immunohistochemistry, which allows the identification of the areas of the tissue where ECM components are being altered [15,31,40]. Taken together, proteomic analysis and these complementary approaches constitute a robust experimental platform to assess the pathological alterations occurring in the ECM as a consequence of the action of SVMPs.



Figure 3. Scheme indicating the different sources of proteins that appear in exudates collected from tissues injected with snake venoms of with isolated SVMPs. After injection in skeletal muscle, viperid venoms or SVMPs induce direct pathological effects, such as degradation of BM components leading to hemorrhage (A); cytotoxicity on various cell types, such as skeletal muscle fibers (B); and degradation of other ECM components. As a consequence of direct tissue damage, resident tissue cells (mast cells, macrophages, fibroblasts) synthesize and release a number of mediators, favoring increments in vascular permeability leading to edema. An inflanumatory infiltrate (C), composed mainly of neutrophils and macrophages, also contributes to the release of proteinases and other mediators. (D) Summary of SVMP-induced damage to muscle fibers and the microvasculature. As a consequence, the exudate that forms in the tissue is composed of proteins uriginating from different sources, as indicated in the bottom of the figure. Magnification in A, B and C: 200 ×.

Table 1. Extracellular matrix proteins detected in exudates collected from mice injected in the gastrocnemius muscle with snake venom metalloproteinases (SVMPs) [15,40,41].

Collagens	
Collagen α -1 (l) chain (lsoform 1) Collagen α -2 (l) chain	
Collagen α-1 (II) chain (Isoform 2)	

Table 1. Cont.

Collagens	
Collagen α-1 (III) chain	
Collagen α-1 (V) chain	
Collagen α-3 (VI) chain	
Collagen α-1 (VII) chain	
Collagen α -2 (XI) chain (Isoform 7)	
Collagen α -1 (XII) chain (Isoform 1)	
Collagen α-1 (XIV) chain (Isoform 1)	
Collagen α-1 (XV) chain	
Collagen α-1 (XVI) chain (Isoform 1)	
Collagen α-1 (XVIII) chain (Isoform 2)	
Collagen α-1 (XIX) chain	
Collagen q-1 (XXII) chain (Isoform 2)	
Collagen α-1 (XXVII) chain	
Collagen α-1 (XXVIII) chain (Isoform 1)	
Laminins	
I aminin subunit a-1	_
Laminin subunit a.3 (Isoform B)	
Laminin subunit 6-1	
Laminin 2-2	
Nidesens	_
Nidogens	_
Nidogen-1	
Nidogen-2	
Proteoglycans	
Decorin	
Lumican	
Perlecan	
Basement membrane—specific heparan sulfate proteoglycan co Biglycan	re proteir
Other extracellular matrix (ECM) proteins	
Fibulin-1 (Isoform C)	
Dystroglycan	
Tenascin X	
Thrombospondin-1	
Thrombospondin-4	
Tetranectin	
Vitronectin	

3. Effects of SVMPs on the BM: Identifying Key Protein Targets of Hemorrhagic Toxins

Disruption of the integrity of microvessels leading to hemorrhage is one of the most important effects induced by viperid SVMPs [11,52,53]. The pioneering ultrastructural studies of McKay et al. [54] and Ownby et al. [36] described drastic alterations in endothelial cells and BM of capillary vessels in tissues injected with hemorrhagic SVMPs. Similar findings were then extended to other SVMPs from different venoms [37,55], and this mechanism of microvessel damage was named "hemorrhage *per rhexis*" [36]. The ability of SVMPs to hydrolyze components of the BM in vitro was demonstrated in several studies [26–32,56]. It was thus hypothesized that hydrolysis of BM components is a key event in the mechanism of hemorrhage by SVMPs.

Proteomic analysis of exudates collected at early time intervals (15 min and 1 h) after injection of crude venom of *Bothrops asper* and several hemorrhagic SVMPs purified from this and other viperid venoms revealed the presence of various BM components, such as laminin, nidogen, type IV collagen, and BM-specific heparan sulfate proteoglycan [15,24,40,41,48], which are the main

components of BMs [38,57–61]. To a large extent, these proteins were degraded, as judged by the molecular mass of the fragments detected in the analyses. The fact that fragments of these BM components were present in exudates collected at early time periods after venom or SVMP injection strongly suggests that hydrolysis of these proteins is due to the direct action of SVMPs in the tissue, in agreement with in vitro observations. Such rapid degradation of BM proteins was corroborated by immunohistochemistry [15,16,31] and Western blotting [15,24,41]. Inhibition of *B. asper* venom with the peptidomimetic hydroxamante metalloproteinase inhibitor Batimastat, prior to injection in mice, resulted in the abrogation of the degradation of BM-specific heparan sulfate proteoglycan core protein (HSPG) [48]. This finding underscores the role of SVMPs in the proteolysis of this proteoglycan as well as a role for HSPG in the stabilization of microvessels.

For years, a puzzling finding regarding the mechanism of action of hemorrhagic SVMPs was that non-hemorrhagic SVMPs were also able to hydrolyze BM-associated proteins in vitro [15,62,63]. This in itself is not particularly surprising as most BM components are susceptible to proteolysis. A comparative analysis of BM degradation by a hemorrhagic and a non-hemorrhagic SVMP from Bothrops sp. venoms contributed to the clarification of this issue. No differences were observed between these enzymes regarding degradation of nidogen and laminin, as judged by proteomic analyses of exudates and by Western blotting of skeletal muscle homogenates [15]. However, a clear distinction occurred when comparing degradation of type IV collagen (by Western blot and immunolistochemistry) and HSPG (by proteomics and Western blot) [15]. In particular type IV collagen is known to play a key role in the mechanical stability of BM owing to the formation of interchain covalent bonds of various types and supramolecular networks between collagen chains [64,65]; these results strongly suggest that the ability of SVMPs to induce hemorrhage is related to their capacity to hydrolyze these BM components. This hypothesis was supported by a study comparing BM degradation by SVMPs of classes I, II, and III, which have a variable domain composition and different intrinsic hemorrhagic activity [24]. The doses of these enzymes injected were adjusted so as to induce the same extent of hemorrhage. In these conditions, there was a similar extent of degradation of type IV collagen and HSPG [24], thus reinforcing the concept that hydrolysis of these components seems to be critical for the onset of microvascular damage and hemorrhage.

The cleavage sites of type IV collagen by a hemorrhagic SVMP from the venom of the rattlesnake *Crotalus atrox* have been determined [23]. The relevance of type IV collagen hydrolysis in the pathogenesis of hemorrhage has been also shown in the case of a PIII SVMP from the venom of *Bothrops jararaca* [16,66,67] and of a hemorrhagic metalloproteinase from the prokaryote *Vibrio vulnificus* [68]. Moreover, genetic disorders affecting type IV collagen are associated with vascular alterations and hemorrhagic stroke [69–71]. HSPG is also known to contribute to the mechanical stabilization of BM in capillary vessels, and embryos having mutations in this proteoglycan show dilated microvessels in the brain and skin, associated with vessel disruption and severe bleedings [72–74]. This agrees with these proteins having a key role in the mechanical stabilization of BMs and therefore on the action of hemorrhagic SVMPs.

The ability of hemorrhagic SVMPs to hydrolyze components that contribute to the mechanical stability of capillaries has been integrated into a 'two-step' hypothesis to explain the mechanism of SVMP-induced hemorrhage [10,52,53]. The first step is the enzymatic hydrolysis of BM components, especially type IV collagen, and also HSPG, with the consequent weakening of the mechanical stability of the BM. Such hydrolysis may also affect cell-cell and cell matrix interactions. Then, the hemodynamic biophysical forces normally operating in the circulation, especially hydrostatic pressure-mediated wall tension and shear stress, cause a distention of the capillary wall, which ends up with the disruption in the integrity of endothelial cells and the vessel wall, with the consequent extravasation.

In addition to capillary BM, SVMPs also affect the BM of other tissue components. The presence of laminin subunit α3 in exudates collected after injection of a PI SVMP [40] suggests that this enzyme degrades laminin at the BM of the dermal-epidermal junction, since this laminin isoform is characteristic of the skin [75,76]. This SVMP induces skin blistering, suggesting that hydrolysis of laminin, and probably other components of the dermal-epidermal interface, is the basis for blister formation. Immunohistochemical observations revealed the presence of laminin in the two sides of the blister, thus supporting the contention of hydrolysis of the BM structure in the skin [40]. Moreover, BM hydrolysis by SVMPs is likely to affect tissue structure, since BM components, especially type IV collagen, are known to play a central role in the organization of tissue architecture [77]. Thus, alterations induced by SVMPs as a consequence of hydrolysis of BM components go beyond the acute effects associated with hemorrhage, blistering, and myonecrosis, since they also affect tissue organization and, probably, cell proliferation and regeneration occurring after tissue damage (Figure 4).



Figure 4. Hydrolysis of ECM components by SVMPs. Some SVMPs hydrolyze components at the BM of capillary vessels, skeletal muscle fibers, and dermal-epidermal junction. In the case of hemorrhagic SVMPs, it has been postulated that hydrolysis of type IV collagen is a key step in the destabilization of BM, which leads to extravasation. SVMPs also hydrolyze additional ECM proteins, such as FACITs, type VI collagen, and other components that connect the BM with the surrounding matrix stromal proteins. Moreover, SVMP degrade proteins that bind to and organize fibrillar collagens, leading to a disorganization of the ECM supramolecular structure. SVMPs may also hydrolyze plasma membrane components, such as integrins, that interact with BM components. All these hydrolytic actions result in a profound alteration of ECM, with consequences for the processes of venom-induced tissue damage, repair, and regeneration.

4. The Action of SVMPs on Proteins that Connect the BM with the Stromal Components of ECM

Proteomic analyses of exudates collected from tissues affected by snake venoms and SVMPs have allowed the detection of degradation products of proteins that play a role in the integration of the BMs with the surrounding ECM (Figure 4). This was a hitherto unknown aspect of venom-induced ECM degradation, since the traditional experimental tools did not allow for the in vivo assessment of hydrolysis of these components. These ECM proteins are essential for the stability and mechanical integration of BMs with other ECM proteins, and for the assembly of fibrillar components of the matrix. For example, type VI collagen is a beaded-filament-forming collagen which integrates BM with fibrillar collagens and other components of the ECM. It interacts with types IV, XIV, I, and II collagens, and with perlecan, decorin, and lumican [78–80], and plays a key role in the mechanical stability of skeletal muscle cells. Deficiencies in type VI collagen have been associated with Ulrich syndrome, a muscle dystrophic condition [81,82] and with other myopathies [83,84]. Degradation products of type VI collagen have been found in exudates collected from tissues after injection of *B. asper* venom and SVMPs of the classes PI, PII, and PIII [15,24,40,41].

The potential implications of hydrolysis of this particular collagen in the action of SVMPs deserve additional consideration as this might affect the mechanical stability of skeletal muscle fibers. The resulting decreased stability of the fibers could contribute to the skeletal muscle pathology initially caused by myotoxic PLA₂s, which affect the integrity of muscle cell plasma membrane [13]. SVMP-induced hydrolysis of type VI collagen, and the consequent weakening of the mechanical stability of muscle BM, together with PLA₂-induced plasma membrane perturbation, may be an example of toxin-toxin synergism to give rise to lesions to the periphery of muscle fibers, leading to myonecrosis. Likewise, by affecting the stability of muscle cell BM, type VI collagen hydrolysis might hamper the process of skeletal muscle regeneration, which depends on the integrity of muscle BM [85,86]. The observation that fragments of type VI collagen are more abundant at early time intervals in exudates from mice injected with *B. asper* venom suggests that such degradation is due to the action of SVMPs [41]. Like laminin, type VI collagen is also involved in the pathogenesis of blistering in snakebite envenomings.

The possibility that hydrolysis of type VI collagen plays a role in the pathogenesis of hemorrhage also deserves discussion. Although the most likely mechanism by which SVMPs induce capillary damage and hemorrhage is their ability to hydrolyze type IV collagen and possibly HSPG at the BM, the degradation of ECM components that link the BM with fibrillar collagens needs to be considered as a possible mechanism of capillary damage as well. The observation that exudate collected from tissue injected with a PI hemorrhagic SVMP contains higher amounts of degradation products of type VI collagen than samples collected from mice injected with a non-hemorrhagic PI SVMP lends support to this hypothesis [15].

Proteomic analyses also identified degradation products of types XII, XIV, and XV collagens in exudates from tissues affected by B. asper venom and SVMPs [15,24,40,41]. Exosites in the Cys-rich domain of SVMPs mediate their interaction with type XII and XIV collagens [89], thus targeting PIII SVMPs to interact and hydrolyze these ECM proteins. These collagens are fibril-associated collagens with interrupted triple helices (FACITs) and play a role in the supramolecular organization of fibrillar collagens [90,91], as well as in the integration of BM with the ECM fibrillar components [92,93]. In skeletal muscle, these FACITs are important for connecting the muscle cell BM with the epimysium and the perimysium [94,95]. The fact that no differences were observed in the amounts of degradation products of types XII and XIV collagens in exudates from tissue injected with hemorrhagic and non-hemorrhagic PI SVMPs [15] argues against a role of hydrolysis of these proteins in the mechanism of hemorrhage. However, the possible involvement of such hydrolysis in the stability of muscle fibers, and on the integration of muscle cells with muscle connective tissue at epimysium and perimysium, has to be considered. Type XV collagen, on the other hand, is a proteoglycan often expressed in BM zones, in regions adjacent to BM where several proteins anchor BM to the subjacent ECM, where it has been proposed to act as a BM organizer [96-98]. Moreover, type XV collagen has a restricted and uniform presence in many tissues, including vascular and muscle BM zones [96,99]. Genetic mutations of this protein in mice have been associated with abnormal capillary morphology, extravasated erythrocytes, and cell degeneration in heart and skeletal muscle [61,100,101]. The relevance of this protein in the organization of the microvasculature has been also demonstrated [101]. Interestingly, as in the case of Type VI collagen, a hemorrhagic SVMP induces higher amounts of Type XV collagen in exudates than a non-hemorrhagic SVMP [15].

The ability of SVMPs to hydrolyze FACITs and proteoglycans having a role in the assembly of fibrillar collagens and in the integration of fibrillar collagens with BMs and other components of the connective tissue has implications for the ability of snake venoms to digest skeletal muscle tissue. The disruption of the connective tissue matrix resulting from the hydrolysis of these integrative components would facilitate the diffusion of snake venom components through the tissues and into the circulation [102]. This action could work in concert with the action of other venom hydrolases, such as hyaluronidase, a well-known spreading factor present in many venoms [35]. This would, in turn, favor the digestive role of SVMPs and venom serine proteinases, as a consequence of the disruption in the organization of muscle tissue. Since viperid venoms are often injected intramuscularly, and since muscle tissue comprises a significant mass of prey, the action of SVMPs on these ECM components is likely to represent a significant contribution to the digestion of muscle mass. Likewise, the 'softening' and disorganization of interstitial connective tissue described above may promote the digestion of the muscle mass of prey by proteinases of the gastric and pancreatic secretions of snakes after ingestion.

From the human pathology standpoint, such disruption of the components of connective tissue may play a role in venom dispersion in the tissues, thus facilitating the systemic action of venom toxins, and also may contribute to the extent of local tissue damage by making the connective tissue more amenable to digestion by endogenous proteinases, such as matrix metalloproteinases (MMPs), which are synthesized as part of the inflammatory response [103,104]. These effects on FACITs and related integrative ECM components may also affect the process of skeletal muscle repair and regeneration, an issue that deserves more investigation.

5. Action of SVMPs on Fibrillar Collagens: A Secondary Outcome of SVMP-Induced Local Tissue Damage

Proteomic analysis of exudates collected from mice injected with venom of B. asper and with hemorrhagic and non-hemorrhagic SVMPs has revealed the presence of degradation fragments of fibrillar collagens, i.e., types I, II, III collagens [15,24,40,41,48]. Since SVMPs are not able to hydrolyze fibrillar collagens lacking triple helical interruptions [105], the basis for this degradation is intriguing. A number of observations strongly suggest that it is due to the action of endogenous proteinases, especially MMPs, which are synthesized and secreted by resident and infiltrating cells in the course of the inflammatory response that follows the acute tissue damage induced by the venom. When comparing exudate proteomics from mice injected with a PI hemorrhagic SVMP to that from tissue injected with a myotoxic PLA2, higher amounts of types I and III collagens were found with the latter. Myotoxic PLA₂s induce muscle necrosis of rapid onset by damaging the integrity of muscle fiber plasma membrane [106] and induce inflammation characterized by pain, edema, synthesis of cytokines and MMPs, and a prominent cellular infiltrate [103,107,108]. Thus, it is suggested that the hydrolysis of fibrillar collagens is a consequence of the action of endogenous MMPs and perhaps other proteinases derived from resident and inflammatory cells. Degradation products of fibrillar collagens were also detected in exudates collected from SVMP-injected muscle [15,24,40,48]. Interestingly, no differences were observed in the amounts of these fragments after injection of hemorrhagic and non-hemorrhagic SVMPs [15], both of which induce an inflammatory response in the tissue.

Additional evidence in support of the concept that hydrolysis of fibrillar collagens is due to endogenous proteinases synthesized during inflammation is that degradation products of types I and III collagens in exudates from mice injected with *B. asper* venom reach their highest amounts in samples collected 24 h after envenoming, whereas type IV collagen products are most abundant in samples collected at 1 h [41]. This suggests that type IV and VI collagens, as well as other BM components and FACITs are hydrolyzed by SVMPs during the early phase of envenoming, whereas fibrillar collagens

are hydrolyzed by endogenous proteinases at later time intervals. In the biological context, such as the case of natural envenomings in prey, this second stage in ECM degradation of interstitial fibrillar collagen is likely to be accomplished by digestive proteinases from gastric and pancreatic secretions of the snakes.

Hydrolysis of ECM Proteins Alters Cell-Matrix Interactions and Generates Fragments with Diverse Physiological and Pathological Actions

In addition to the direct pathological consequences of degradation of ECM by SVMPs, another important consequence of this hydrolysis is the alteration of the interaction between ECM and cells. For instance, fibronectin interacts with cells through the integrin $\alpha 5\beta 1$, and is involved with other extracellular signals to regulate morphogenesis and cellular differentiation [109]. SVMPs hydrolyze fibronectin in vitro [26,27,30] and fibronectin degradation products are detected in the proteomics analysis of exudates of tissues injected with SVMPs [24,40]. Although plasma fibronectin is probably present in exudates as a consequence of increments in vascular permeability, it is very likely that ECM fibronectin is also hydrolyzed and contributes to fragments in exudates.

SVMP-induced ECM degradation may also release proteins or protein fragments that exert a variety of physiological effects. For instance, hydrolysis of types XV and XVIII collagens results in the generation of endostatin, an inhibitor of angiogenesis [110–112], and the cleavage of the α 3 chain of type IV collagen by MMPs releases the fragment tumstatin, which is a potent anti-angiogenic molecule [113,114]. Another BM-derived fragment is endorepelin, the C-terminal fragment of perlecan, which also exerts anti-angiogenic activity [115]. Since SVMPs release fragments of all of these proteins in the exudates [15,24,40,41,48], it is suggested that some of them exert anti-angiogenic activity and influence the tissue repair process. SVMPs may also release matrikines from ECM proteins which regulate a number of cellular activities [116]. Our own studies indicate that snake venom and SVMPs release a number of DAMPs in the affected tissues, which are likely to play diverse roles in the processes of tissue damage and repair (unpublished results). Another interesting protein that has been found elevated in exudates collected from mice injected with B. asper venom and SVMPs is thrombospondin-1 [15,41,48], a counter-adhesive protein that influences endothelial cell behavior by modulating cell-matrix and cell-cell interactions and by regulating growth factors [117]. This protein has been shown to play roles in hemostasis, inflammation, tissue regeneration, and angiogenesis [118–121]. Therefore, the release of this protein into the exudate by SVMPs could modulate the inflammatory process and the consequent tissue repair response.

BM and other ECM components act as storage sites for a variety of growth factors and other physiologically-active components, such as insulin growth factor (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF) [122]. Proteolytic processing of ECM components in inflammation releases growth factors thus influencing cell activation, differentiation, and proliferation [123]. Regarding angiogenesis, the action of SVMPs might result in the release of both pro-angiogenic, e.g., VEGF, and anti-angiogenic, e.g., endostatin and endorepelin, components. Hence, hydrolysis of ECM by SVMPs is likely to result in the release of diverse mediators, which in turn may expand tissue alterations, dysregulate cell-matrix interaction, promote and inhibit cell proliferation, and play reparative and regenerative roles in the complex tissue interactive landscape. This is an aspect of SVMP-induced ECM alterations that needs to be explored in detail.

Hydrolysis of ECM components might also result in changes in the mechanical properties of the matrix and on the interaction of ECM and cells. It is known that the stiffness of ECM varies depending on many factors, and that changes in such stiffness bring consequences for cellular behavior in many ways, including cell differentiation [124]. Likewise, the release of growth factors stored in the matrix may occur not only by direct proteolysis, but also by mechanical forces generated in the ECM as a consequence of hydrolysis by proteinases [125–127]. The biomechanical consequences of ECM degradation by SVMPs constitute an area of research that needs to be developed, since phenomena

associated with changes in cellular behavior secondary to mechanical alterations in the matrix may have consequences in the processes of tissue inflammation, repair, and regeneration.

7. Concluding Remarks

The proteomic analysis of exudates collected from mice injected with snake venoms and isolated toxins has opened an avenue to study hitherto unknown aspects of the action of venom enzymes on the ECM, an issue that has been largely elusive in toxinological research. This methodological platform, when combined with histological, ultrastructural, immunohistochemical, and immunological methods, has provided new and valuable information on the pathogenesis of tissue damage induced by viperid snake venoms.

The studies reviewed in this communication uncovered a pathophysiological scenario characterized by various levels of ECM degradation by SVMPs. BM components are rapidly hydrolyzed upon venom and SVMP injection in the tissues. Of special significance for the pathogenesis of microvascular damage leading to hemorrhage is the hydrolysis of structurally-relevant BM components, especially type IV collagen and, possibly, HSPG. In addition to microvessels, hydrolysis of specific BM components detected in exudate could also affect the stability of skeletal muscle fibers as well as the muscle regenerative process. It can also be postulated that BM damage affects the spatial organization of cells in the tissue owing to the role of this ECM structure in the compartmentalization of cells, in addition to favoring the spread of venom components in the tissue and to the circulation. Figure 5 summarizes the main effects of SVMPs on the ECM.

Concomitantly, SVMPs also hydrolyze ECM proteins that play a role in the integration of BM with the surrounding matrix, and also in the assembly of fibrillar collagens in the matrix, such as types VI, XII, XV, and XIV collagens. The hydrolysis of these FACITs and related proteins may contribute to the collapse of BM, but also may result in the disorganization of the interstitial fibrillar matrix, favoring tissue disorganization, venom spreading, and digestion. On the other hand, the observed hydrolysis of fibrillar collagens I and III is likely to be a consequence of the action of endogenous proteinases, especially MMPs, and not to the direct action of SVMPs. Hence, as part of the inflammatory process that ensues as a consequence of venom induced acute tissue damage, MMPs and other endogenous proteinases, derived from resident tissue cells or invading leukocytes, hydrolyze fibrillar collagens, resulting in widespread ECM degradation, which further complicates venom-induced tissue damage. Moreover, hydrolysis of ECM provides protein fragments of diverse physiological actions that are likely to participate in tissue alterations, as well as in inflammation, repair, and regeneration. Likewise, biomechanical changes in the tissue occurring as a result of changes in the stiffness of ECM after SVMP action may affect the behavior of cells.

Understanding the mechanisms involved in ECM degradation in snakebite envenoming may pave the way for the search of novel therapeutic agents, aimed at the inhibition of SVMPs and at the modulation of the inflammatory response. A rapid administration of SVMP inhibitors in the field, in combination with the use of anti-inflammatory agents and the antivenom, may contribute to ameliorate the extent of local tissue damage and hence the magnitude of the sequelae in people envenomed by viperid snakebites. In addition, a deeper understanding of venom-induced ECM damage may provide information for designing interventions aimed at reducing snakebite envenoming morbidity by improving the processes of tissue repair and regeneration.



Figure 5. Summary of the effects induced by SVMPs on the ECM components. SVMPs hydrolyze components of the BM (type IV collagen, laminin, nidogen, heparan sulfate proteoglycan core protein (HSPG)) in capillary blood vessels, skeletal muscle fibers and dermal-epidermal junctions. As a consequence, hemorrhage and blistering occurs, and it is hypothesized that acute skeletal muscle damage also ensues. On the other hand, hydrolysis of FACITs, type VI collagen and other components results in alterations in the interactions between BM and the surrounding stromal components. In addition, hydrolysis of ECM proteins results in exposition of cryptic sites, release of growth factors stored in the matrix, and generation of a variety of protein fragments with potent biological activities, which are involved in pathological, reparative, and regenerative events.

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