SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 10
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
MARGARITA CASTELLANOS ABONDANO

Asunto: Radicación: 11-180116- -4
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 5159
Folios: 10

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/JP2010/059953				
Fecha de Presentación Internacional	11/06/2010				

1. Documentos estudiados

Descripción:	Radicación 11-180116-00000	28/Diciembre/2011
Reivindicaciones:	Radicación 11-180116-00000	28/Diciembre/2011
Prioridad(es):	JP2009-140585	11/Junio/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque su contenido técnico corresponde con el de la prioridad reivindicada (Art. 9 D 486) y fue presentada dentro del plazo establecido por la D. 486.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las

cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 19 del capítulo reivindicatorio.

Título de la solicitud: “Aptámero para quimasa y su uso”.

El problema planteado en esta solicitud consiste en reducir la actividad de quimasa involucrada en procesos inflamatorios causados por la estimulación de los mastocitos, tal es el caso de fibrosis por adhesión a órganos y enfermedades alérgicas y además, inhibir su capacidad de escindir enlaces peptídicos en a.a. aromáticos como el de angiotensina I para producir angiotensina II y así evitar efectos vasoconstrictores y enfermedades cardiovasculares.

Para resolver este problema técnico la solicitud en estudio proporciona un aptámero capaz de unirse a quimasa que presenta la fórmula: X1GAUAGAN1N2UAAX2.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N15/09, C12Q1/68, A61K31/7088, A61P9/00, A61P19/04, A61P43/00.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

La reivindicación 14 hace referencia al uso del agente farmacéutico. Las reivindicaciones referidas a uso no son patentables en los términos del art. 14 D. 486.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 se define exclusivamente en términos funcionales, se invita a la solicitante a definir la estructura del aptámero por medio de la secuencia de nucleótidos que la codifica e indicándolo desde la reivindicación independiente.

La reivindicación 2 define la secuencia de nucleótidos SEQ ID NO. 21 en la forma de una estructura Markush de tipo:

$$X_1GAUAGAN_1N_2UAAX_2$$

Se invita a la solicitante a introducir la estructura general en la reivindicación independiente con el fin de cumplir con la definición funcional y estructural desde la reivindicación independiente.

Las reivindicaciones 5 y 7 se definen en términos del resultado que se pretende alcanzar con la modificación de al menos uno de los nucleótidos pirimidínicos o en términos generales de la modificación o alteración. Se recuerda a la solicitante que la definición del objeto en términos del resultado que se pretende alcanzar no es admisible porque la reivindicación no solo incluiría el objeto que ha sido sintetizado

sino todas las alternativas presentes o futuras que cumplan la misma condición. Se sugiere retirar la reivindicación.

La reivindicación 6 define el aptámero por referencia a las secuencias de nucleótidos definidas en los literales (a), (b) y (c), donde el objeto reivindicado consiste en un aptámero seleccionado a partir de las SEQ ID NO.:

(a) 4 – 34, 38 – 57, 59 -65 y 69 – 72 con la condición de que uracilo puede ser timina
(b) 4-34, 38-57, 59 – 65 y 69 – 72 con la condición de que el uracilo puede ser timina y en donde 1 a 5 nucleótidos están sustituidos, suprimidos, insertados o adicionados
(c) Una secuencia que tiene una identidad de 70% o más seleccionada de SEQ ID NO. 4-34, 38-57, 59 – 65 y 69-72 con la condición de que uracilo puede ser timina.

Al respecto es pertinente señalar que la reivindicación presenta falta de concisión porque repite de manera innecesaria en los literales (a), (b) y (c) las SEQ ID NO. 4 – 34, 38 – 57, 59 -65 y 69 – 72 que comprenden aproximadamente 72 a 76 nucleótidos y que comparten el sitio activo de 13 nucleótidos definido por la SEQ ID NO. 21. Adicionalmente el literal (b) define las SEQ ID NO. por referencia al resultado que se pretende alcanzar, esto es en términos generales de una posible sustitución, deleción, inserción o adición de nucleótidos sin indicar las posiciones en las que se puede modificar la estructura. Dicha definición no resulta admisible por cuanto incluye todas las alternativas presentes o futuras que cumplan con la misma condición, se sugiere eliminar dichas referencias generales. Asimismo, se sugiere eliminar la referencia a porcentajes de identidad definida en el literal (c), por cuanto no existe sustento ni ejemplificación alguna para fundamentar modificaciones en al menos el 30% de la estructura del aptámero, esto es aproximadamente de 21 nucleótidos.

Por su parte, la reivindicación 8 define el aptámero por referencia a los literales (a´), (b´) y (c´) en las que se contemplan las modificaciones puntuales sobre la SEQ ID NO. 56, 61, 69 y 72 sobre los siguientes nucleótidos: 56(1)-56(7), 56(9), 56(11), 56(12), 56(15)-56(52), 61(1), 69(1), 69(2) y 72(1)-72(8) con la condición de que el uracilo puede ser timina. Por su parte, los literales (b´) y (c´) repiten de manera innecesaria porque hacen referencia a las mismas SEQ ID NO. definidas en el literal (a) indicando la posibilidad de sustituciones, deleciones, inserciones o adición de nucleótidos o por referencia a porcentajes de identidad. En el caso del literal (b) la definición general de las modificaciones constituye un resultado a alcanzar porque no indica las posiciones concretas donde opera la modificación y por otra parte, la referencia a porcentaje de identidad comprende modalidades que no se encuentran ejemplificadas, dado que el 30% de variabilidad en las SEQ ID NO. 56, 61, 69 y 72 que tienen una longitud aproximada de 24 nucleótidos implica al menos modificaciones en al menos 7 nucleótidos y estas modalidades no se encuentran debidamente sustentadas y ejemplificadas en la descripción, por lo anterior se sugiere realizar los ajustes necesarios al capítulo reivindicatorio.

Las reivindicaciones 11 y 12 definen un complejo – conjugado- obtenido por la reacción entre el aptámero y una sustancia funcional de afinidad, marcación, una enzima o bien un vehículo. La caracterización en términos generales que presentan dichas reivindicaciones implican interacciones de tipo electrostático –puentes de

hidrógeno, fuerzas de Van der Wals – de tipo químico .- enlaces covalentes- y de tipo físico al hacer referencia a vehículos para administración o formulación de sistemas de entrega. Dichas reivindicaciones no cuentan con el sustento debido en la descripción por cuanto no existe ningún ejemplo específico para la obtención de complejos, por lo anterior se sugiere retirarlas del capítulo reivindicatorio.

Las reivindicaciones 13 y 14 se encabezan en términos de agente pero hacen nuevamente referencia al complejo. Se sugiere indicar si el término agente corresponde a una composición farmacéutica o a un complejo tipo conjugado y definirlo en tal caso por sus características esenciales, es decir, en el primer caso por sus ingredientes y proporciones y en el segundo por las estructuras moleculares que forman el complejo. Lo anterior sin perjuicio de lo indicado en párrafos anteriores en cuanto a la falta de sustento.

La reivindicación 15 hace referencia al aptámero definido en la reivindicación 2. Sin embargo el encabezado corresponde a la función que busca desempeñar como reactivo de diagnóstico y no se incorporan elementos estructurales adicionales. Por su parte, la reivindicación 16 se encabeza en términos de una sonda de detección de quimasa definida solamente por referencia al aptámero. Al respecto es pertinente señalar que las reivindicaciones no se deben definir o encabezar exclusivamente por referencia a la función y debido a que en los dos casos nuevamente se hace referencia al aptámero previamente definido se concluye que dichas reivindicaciones repiten materia de manera innecesaria y por otra parte se recuerda a la solicitante que las aplicaciones técnicas o ventajas no deben ser incluidas en las reivindicaciones y que sólo se circunscriben a la descripción de la invención.

La reivindicación 17 se encabeza en términos de un portador de fase sólida para purificación de quimasa por referencia exclusiva al aptámero o al complejo. Sin embargo no se presenta la definición de la estructura del portador y dado que en el caso del complejo tampoco hay certeza de si corresponde a una composición o a un conjugado se hace necesario que se presenten las aclaraciones correspondientes.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la prioridad reivindicada: Junio 11 de 2009 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Ítem	Documento	Título	Fecha de Publicación
D1	Zhao, Xiao-Yan, et. al.	“Chymase induces profibrotic response via transforming growth factor-β1/Smad activation in rat cardiac fibroblasts”	05/Diciembre/2007
D2	US5660985	“High affinity nucleic acid ligands containing modified nucleotides”	26/Agosto/1997
D3	WO2005113786	“Diagnosis and therapeutics for diseases associated with chymse (CMA1)”	01/Diciembre/2005

El documento D1 divulga la actividad de la serin-proteasa quimasa 1 (CMA1),

contenida en los gránulos secretores de los mastocitos, en la conversión de angiotensina II en la forma Angiotensina I y su capacidad de clivar el factor de crecimiento TGF-β1 a una forma activa y su rol en la patofisiología de condiciones asociadas al sistema cardiovascular (Pág. 159).

El documento D2[1] divulga ligandos tipo ácido nucleico que incluyen nucleótidos modificados y que son candidatos para enlazarse de forma selectiva a enzimas biológicas. El documento enseña de forma específica el proceso SELEX (Resumen).

El documento D3[2] divulga la posibilidad de obtener análogos de CMA-1 por medio de librerías combinatorias (Pág. 30, líneas 28 y 29). El documento divulga además la posibilidad de obtener oligonucleótidos antisentido de secuencia DNA o RNA capaces de generar inhibición de la quimasa por plegamiento tridimensional (Pág. 27, líneas 19 a 27).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D 486)

La reivindicación 1 consiste en un aptámero que se une a quimasa para inhibir la actividad de quimasa.

Tabla 1.
Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D3
Aptámero	Análogos de CMA-1 (Pág. 27, líneas 19 a 27)
Que se une a quimasa para inhibir la actividad quimasa	Oligonucleótidos antisentido plegados tridimensionalmente como inhibidores de quimasa 1.

Las características esenciales del aptámero definido en la reivindicación 1 se encuentran anticipadas por el documento D3 que divulga oligonucleótidos plegados tridimensionalmente que se enlazan al sitio activo de quimasa 1 (CMA1) y por lo tanto inhiben su actividad y capacidad de hidrolizar angiotensina I para obtener angiotensina (Pág. 28, Líneas 19 a 27).

En consecuencia, la reivindicación 1 no es nueva considerando las enseñanzas del documento D3.

Nivel Inventivo (Art 18 D 486)

La reivindicación 1 consiste en un aptámero que se une a quimasa para inhibir la

1 http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=5660985A&KC=A&FT=D&ND=3&date=19970826&DB=EPODOC&locale=en_EP
2 http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20051201&CC=WO&NR=2005113786A2&KC=A2

actividad de quimasa.

Tabla 2.
Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D3
Aptámero	CMA 1 (Pág. 159)	Análogos de CMA-1 (Pág. 27, líneas 19 a 27)
Que se une a quimasa para inhibir la actividad quimasa		Oligonucleótidos antisentido plegados tridimensionalmente

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga la actividad de la quimasa 1 (CMA 1) y su intervención en la progresión de enfermedades cardiovasculares.

La diferencia entre la invención definida en la reivindicación 1 y las enseñanzas del documento D1 consiste en que la invención hace referencia a un aptámero que se enlaza al sitio activo de la quimasa que es una secuencia de oligonucleótido plegada, capaz de inhibir la actividad de la quimasa (CMA1).

El efecto técnico que se origina al diseñar el aptámero consiste en obtener alternativas terapéuticas para inhibir la actividad de la quimasa 1, en particular un tratamiento biológico alternativo para enfermedades cardiovasculares.

Por lo tanto, el problema técnico objetivo que pretende resolver la invención consiste en: cómo diseñar inhibidores de quimasa CMA1 de tipo oligonucléotidos.

Sin embargo, el documento D3 divulga análogos estructurales de quimasa (CMA1) que corresponden a oligonucleótidos antisentido plegados tridimensionalmente (Pág. 27 líneas 19 a 28). En consecuencia, la persona del oficio normalmente versada en la materia contaba con la sugerencia o motivación razonable para diseñar inhibidores de la actividad de quimasa 1, conociendo su estructura como lo divulga el documento D1, y de diseñar bibliotecas de nucleótidos plegados tridimensionalmente tipo aptámero como lo divulga el documento D3 para el tratamiento de enfermedades cardiovasculares, por lo cual, la reivindicación 1 se considera obvia.

La reivindicación 2 consiste en un aptámero que se une a quimasa para inhibir la actividad de quimasa caracterizado además porque comprende una secuencia de nucleótidos representada por $X_1GAUAGAN_1N_2UAAX_2$ (SEQ ID NO: 21; cada uno de X_1 y X_2 sea idéntico o no, es A o G, y cada uno de N_1 y N_2, sea idéntico o no, es A, G, C, U o T).

Tabla 3.
Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Aptámero	Ligandos de ácido nucleico obtenidos por	Análogos de CMA-1 (Pág. 27, líneas 19

Características esenciales	D2	D3
	el método SELEX (Resumen)	a 27)
Que se une a quimasa para inhibir la actividad quimasa	No menciona	Oligonucleótidos antisentido plegados tridimensionalmente
secuencia de nucleótidos reresentada por $X_1GAUAGAN_1N_2UAAX_2$	No menciona	
SEQ ID NO. 21	No menciona	No menciona

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga ligandos de ácido nucléico obtenidos por el método SELEX capaces de inhibir la actividad enzimática (Resumen).

La diferencia entre la invención definida en la reivindicación 2 y las enseñanzas del documento D2 consiste en que el documento no divulga la estructura del aptámero, oligonucleótido plegado, definida por la estructura general: $X_1GAUAGAN_1N_2UAAX_2$ y el aptámero específico de SEQ ID NO. 21.

El efecto técnico que se origina al diseñar el aptámero consiste en que se produce la inhibición de la actividad de la quimasa 1 (CMA1), producida por los mastocitos, con el fin de detener la conversión de angiotensina I a angiotensina II y por lo tanto la incidencia de enfermedades cardiovasculares.

Por lo tanto, el problema técnico objetivo que pretende resolver la invención consiste en: cómo diseñar aptámeros inhibidores de la actividad de quimasa 1 (CMA1) estructuralmente cercanos al sitio activo de la enzima.

Sin embargo, el documento D3 divulga la posibilidad de diseñar oligonucleótidos con plegamiento tridimensional determinado y obtenidos por librerías combinatorias con el fin de inhibir la actividad de quimasa 1 (Pág. 28 líneas 19 a 27). En consecuencia, la persona versada en la materia se encontraba en capacidad de deducir la estructura del sitio activo de quimasa 1, con base en las enseñanzas del documento D3 y de utilizar el método SELEX para obtener bibliotecas de oligonucleótidos con plegamiento específico capaz de inhibir la actividad de la enzima, como lo divulga el documento D2, razón por la cual la materia definida en la reivindicación 2 se considera obvia y sin nivel inventivo.

Puesto que las reivindicaciones 2 a 17 no divulgan elementos característicos adicionales, se considera que dichas reivindicaciones tampoco cumplen con el requisito de nivel inventivo.

La reivindicación 6 consiste en un aptámero que se une a quimasa para inhibir la actividad de quimasa caracterizado además porque comprende cualquiera de las secuencias de nucleótidos (a), (b) y (c) siguientes: (a) 4 – 34, 38 – 57, 59 -65 y 69 – 72 con la condición de que uracilo puede ser timina, (b) 4-34, 38-57, 59 – 65 y 69 – 72 con la condición de que el uracilo puede ser timina y en donde 1 a 5 nucleótidos

están sustituidos, suprimidos, insertados o adicionados y (c) una secuencia que tiene una identidad de 70% o más seleccionada de SEQ ID NO. 4-34, 38-57, 59 – 65 y 69-72 con la condición de que uracilo puede ser timina.

Tabla 4.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Aptámero	Ligandos de ácido nucleico obtenidos por el método SELEX (Resumen)	Análogos de CMA-1 (Pág. 27, líneas 19 a 27)
Que se une a quimasa para inhibir la actividad quimasa	No menciona	Oligonucleótidos antisentido plegados tridimensionalmente
comprende cualquiera de las secuencias de nucleótidos (a), (b) y (c) siguientes	No menciona	No menciona
(a) 4 – 34, 38 – 57, 59 -65 y 69 – 72 con la condición de que uracilo puede ser timina	No menciona	No menciona
(b) 4-34, 38-57, 59 – 65 y 69 – 72 con la condición de que el uracilo puede ser timina y en donde 1 a 5 nucleótidos están sustituidos, suprimidos, insertados o adicionados y	No menciona	No menciona
(c) una secuencia que tiene una identidad de 70% o más seleccionada de SEQ ID NO. 4-34, 38-57, 59 – 65 y 69-72 con la condición de que uracilo puede ser timina	No menciona	No menciona

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 6 porque divulga ligandos de ácido nucléico obtenidos por el método SELEX capaces de inhibir la actividad enzimática (Resumen).

La diferencia entre la invención definida en la reivindicación 6 y las enseñanzas del documento D2 consiste en que el documento no divulga la estructura del aptámero completo, es decir del oligonucleótido plegado que consiste en alrededor de 73 nucleótidos y que se define por las SEQ ID NOs. 4-34, 38-57, 59 – 65 y 69 -72.

El efecto técnico que se origina al diseñar el aptámero completo análogo de quimasa 1 consiste en que se produce la inhibición de la actividad de la quimasa 1 (CMA1), producida por los mastocitos, con el fin de detener la conversión de angiotensina I a angiotensina II y por lo tanto la incidencia de enfermedades cardiovasculares.

Por lo tanto, el problema técnico objetivo que pretende resolver la invención consiste en: cómo diseñar aptámeros inhibidores de la actividad de quimasa 1 (CMA1).

Sin embargo, el documento D3 divulga la posibilidad de diseñar oligonucleótidos con

plegamiento tridimensional determinado y obtenidos por librerías combinatorias con el fin de inhibir la actividad de quimasa 1 (Pág. 28 líneas 19 a 27). En consecuencia, la persona versada en la materia se encontraba en capacidad de deducir la estructura del sitio activo de quimasa 1, con base en las enseñanzas del documento D2 y de utilizar el método SELEX para obtener bibliotecas de oligonucleótidos con plegamiento específico capaz de inhibir la actividad de la enzima, como lo divulga el documento D2, razón por la cual la materia definida en la reivindicación 6 se considera obvia y sin nivel inventivo.

Puesto que las reivindicaciones 7 a 17 no divulgan elementos característicos adicionales, se considera que dichas reivindicaciones tampoco cumplen con el requisito de nivel inventivo.

8. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Zhao, Xiao-Yan, et. al.	1 a 17	Nivel Inventivo
D2	US5660985	1 a 17	Nivel Inventivo
D3	WO2005113786	1 a 17	Novedad/Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

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El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-05-06

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Chymase induces profibrotic response via transforming growth factor-β1/Smad activation in rat cardiac fibroblasts

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Abstract Mast cell-derived chymase is implicated in myocardial fibrosis (MF), but the underlying mechanism of intracellular signaling remains unclear. Transforming growth factor-β1 (TGF-β1) is identified as the most important profibrotic cytokine, and Smad proteins are essential, but not exclusive downstream components of TGF-β1 signaling. Moreover, novel evidence indicates that there is a cross talk between Smad and mitogen-activated protein kinase (MAPK) signaling cascade. We investigated whether chymase activated TGF-β1/Smad pathway and its potential role in MF by evaluating cardiac fibroblasts (CFs) proliferation and collagen synthesis in neonatal rats. MTT assay and ^{3}H-Proline incorporation revealed that chymase induced CFs proliferation and collagen synthesis in a dose-dependent manner. RT-PCR and Western blot assay demonstrated that chymase not only increased TGF-β1 expression but also upregulated phosphorylated-Smad2/3 protein. Furthermore, pretreatment with TGF-β1 neutralizing antibody suppressed chymase-induced cell growth, collagen production, and Smad activation. In contrast, the blockade of angiotensin II receptor had no effects on chymase-induced production of TGF-β1 and profibrotic action. Additionally, the inhibition of MAPK signaling had no effect on Smad activation elicited by chymase. These results suggest that chymase can promote CFs proliferation and collagen synthesis via TGF-β1/Smad pathway rather than angiotensin II, which is implicated in the process of MF.

Keywords Chymase · Cardiac fibroblasts · Myocardial fibrosis · Transforming growth factor-β1 · Smad · Rat

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Introduction

Myocardial fibrosis (MF) is a consequence of various cardiovascular diseases [1–3] and characterized by proliferation of cardiac fibroblasts (CFs) and excessive deposition of extracellular matrix collagen [4, 5]. It has long been considered that MF is a major determinant and common pathway leading to end-stage heart failure regardless of the initial cause of the disease [2, 3, 6, 7]. Therefore, there has been growing interest in the investigation of mechanism underlying fibrosis in recent years [1–5]. Nowadays, chymase, a chymotrypsin-like serine protease which is contained in the secretory granules of the mast cells, has attracted a great deal of attention with regard to the conversion of angiotensin II (Ang II) from Ang I [8–11] and the cleavage of latent transforming growth factor-β1 (TGF-β1) to an active form [12]. Chymase is relatively abundant in mammalian hearts, including human and rat hearts [8, 11], and also has been found to play a central role in many pathophysiological conditions of cardiovascular system [8–11, 13–15]. Although, it is

commonly accepted that mast cell chymase-dependent Ang II pathway is associated with cardiac remodeling process [8–10], the relative role of chymase-mediated TGF-β1 forming system in MF has not been well established.

A significant body of literature demonstrates that TGF-β1, a key mediator responsible for MF in both experimental and human cardiac diseases [2, 3, 5, 16–18], exerts pronounced effects on fibroblast proliferation [19–22] and extracellular matrix production in vitro [5, 23]. Moreover, a major advance in understanding TGF-β1 post-receptor signaling is the identification of Smad proteins [24, 25] despite novel evidence suggests that there are Smad-independent pathways in TGF-β signaling recently [24, 26]. Whether chymase-dependent TGF-β1 formation contributes to activation of Smad signaling and its profibrotic effects in CFs are still poorly understood. Further insight into the mechanism may have important therapeutic implications.

The present study thereby was to investigate the direct effects of chymase on cell proliferation and collagen synthesis, and its intracellular signaling transduction in rat CFs. Our results reveal a novel mechanism by which chymase can activate TGF-β1/Smad pathway and thus exert an important effect on MF through facilitating CFs growth and collagen synthesis.

Materials and methods

Cell culture

All experimental protocols were approved by the Animal Experiments Committee of the Fourth Military Medical University. CFs were isolated and cultured from 1- to 3-day-old neonatal Sprague–Dawley rats as described previously [27]. All experiments were performed on Passage 2–3. The purity of CFs was greater than 98% on the basis of positive staining for vimentin and negative staining for α-smooth muscle actin and von Willebrand factor. Studies were conducted on CFs, which were grown to 80% confluence and serum-starved for 48 h in serum-free medium before treatment. Then the CFs were treated with different reagents either alone or co-incubation for designated times in the presence of 1% fetal bovine serum (FBS).

Cell proliferation assay

Tetrazolium (3-(4,5-dimcthyl thioazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) assay was performed to measure cell numbers. CFs were seeded in a 96-well plate at a density of 0.5×10^4 cells/well. The serum-starved cells were unstimulated or stimulated with chymase (7.5–30 ng/ml) for 24 h. Some cells were pretreated with chymase inhibitor (10 μM), or TGF-β1 neutralizing antibody (50, 100 μg/ml), AT1 blocker valsartan (10 μM), AT2 blocker PD123319 (10 μM) for 1 h, and incubated for 24 h in the presence of 30 ng/ml chymase. Afterwards, 20 μl of 5% MTT solution was added in each well and incubated with cells for 4 h. The supernatant was aspirated and then 150 μl of DMSO was added. Absorbance (*A*) value was measured at 490 nm of wavelength with enzyme-linked immunoassay system.

Collagen synthesis assay

The collagen synthesis was determined by measuring ^{3}H-Proline incorporation. In brief, CFs were plated on 24-well culture dishes at a density of 3×10^4 cells/well, and treated with or without different reagents as mentioned above. The cells were labeled with ^{3}H-Proline (4 μCi) for the last 4 h, then fixed in 10% trichloroacetic acid, and lysed in 0.3 N NaOH. Aliquots from four wells for each treatment with 1 ml scintillation fluid were counted in a liquid scintillation counter. The data were normalized to cell numbers.

RNA isolation and RT-PCR

The expression of TGF-β1 mRNA was determined by RT-PCR assay. At the end of the incubation, total RNA was extracted following the established method [28]. Complementary DNA was synthesized from 2 μg of total RNA with AMV reverse transcriptase kit (Promega, WI) according to the manufacturer's instruction. Then PCR amplifications were performed. All samples were subjected to RT-PCR for housekeeping gene GAPDH as an internal standard. The sequences of the specific primers were as follows: TGF-β1 (531 bp) 5′-GAC TCC TGC TGC TTT CTC C-3′ (sense); 5′-GCG GTC CAC CAT TAG CAC-3′ (antisense); GAPDH (231 bp) 5′-ACG GCA AAT TCA ACG GCA CAG TCA-3′ (sense); 5′-TGG GGG CAT CGG CAG AAG G-3′ (antisense). The mixture was heated initially for 5 min at 94°C followed by 35 cycles (45 s at 94°C, 30 s at 60°C, and 45 s at 72°C). A final extension was performed at 72°C for 10 min. Afterwards, the PCR products were separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide. The bands were visualized and quantificated with image analysis software (Quantity One, Bio-Rad).

Protein extraction and assay

Cells were seeded on 6-well plates at a density of 5×10^4 cells/well and untreated or treated with different

reagents for indicated periods. At the end of the incubation, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 2 mM EDTA; 0.1% SDS) in the presence of protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF) for 30 min on ice. Total lysates were centrifuged (14,000*g*, 4°C, 10 min) to pellet cell debris. The supernatant fraction was stored at −80°C and used for the protein determination of TGF-β1, Smad7 and Smad2/3. For phosphorylated-Smad2/3 protein detection in nuclear fraction, the isolated nuclei from CFs, using the Nuclear Isolation Kit (Sigma-Aldrich, Canada) according to the manufacturer's instructions, were resuspended in the above lysis buffer adding 0.1% Triton X-100 and phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, and 20 mM β-glycorophosphate). This lysate was subject to sonication for 5 s (repeated five times) to disrupt nuclear membranes. The subsequent procedure was the same as mentioned above. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL).

Western blot analysis

Prestained low-molecular-weight marker and 20 μg of protein from samples were subject to 10% SDS-PAGE. Separated protein was transferred onto PVDF membranes which was blocked in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk powder for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4°C. The blots were rinsed in TBS-T, and incubated with secondary antibodies for 2 h at room temperature. Bands on Western blots were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham International, Arlington Heights, IL) according to the manufacturer's protocol. Optical densities of the bands were scanned and quantified with image analysis software (Quantity One, Bio-Rad).

Isolation and purification of mast cell chymase

Serosal mast cells were isolated from the pleural and peritoneal cavities of rats and stimulated to exocytose their chymase-containing cytoplasmic granules, as described previously [29]. Rat chymase was purified from the isolated granule remnants to apparent homogeneity according to the Refs. [12, 30]. To verify the purity of chymase, the enzyme was alkylated with 4-vinylpyridine and further purified by reversed-phase (RP) high-performance liquid chromatography (HPLC) on a 0.21 × 15.0 cm column (Poros R2, PerSeptive Biosystems). The chymase, which eluted from the RP column as a single peak both before and after alkylation, was then dried and digested with trypsin. The enzyme was electrophoresed on a SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). Amino terminal sequencing of the membrane containing electroblotted enzyme was performed on a protein sequencer (Applied Biosystems model 477A). The purity of chymase is more than 95% by SDS-PAGE, and its specific activity was 1 unit/μg when measured spectrophotometrically (Unit Definition: One unit will hydrolyze 1.0 μM of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) per minute at pH 7.8 at 25°C).

Reagents

All reagents for cell culture were purchased from Gibco-BRL (MA, USA). Antibodies raised against the following proteins were used: TGF-β1, Smad7, β-Actin and HRP-conjugated secondary antibodies (Santa Cruz, CA), P-Smad2/3 (Upstate Biotechnology, Lake Placid, NY), Smad2/3 (Cell Signaling Technology, Beverly, MA). TGF-β1 neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). The chymase inhibitor Suc-Val-Pro-Phe^{P}(OPh)$_2$ and other laboratory grade reagents were from Sigma (Saint Louis, MO).

Statistical analysis

The data are presented as means ± SD. Statistical differences between groups were assessed by one-way ANOVA, followed by the LSD post hoc test. Differences were considered statistically significant at $P < 0.05$.

Results

Chymase promotes CFs proliferation and collagen synthesis

The effects of chymase on cell proliferation and total collagen synthesis of CFs were determined by MTT assay and ^{3}H-Proline incorporation. As shown in Fig. 1A, B, treatment with chymase (15–30 ng/ml) for 24 h significantly increased both A_{490} Value and incorporation of ^{3}H-Proline in a dose-dependent manner, whereas no significant difference was shown at 7.5 ng/ml compared with the control ($P > 0.05$). To further confirm these changes were induced by chymase, we observed the effects of Suc-Val-Pro-Phe^{P}(OPh)$_2$, a chymase inhibitor (CHI), on the chymase-stimulated profibrotic response. Compared with the

30 ng/ml chymase group, the A_{490} Value and ^{3}H-Proline incorporation were reduced by 38% and 35%, respectively, when these cells were pretreated with 10 μM CHI (Fig. 1A, B). However, CHI (10 μM) alone had no significant influence on these effects (data not shown).

Chymase increases TGF-β1 mRNA and protein expressions in CFs

The mRNA and protein levels of TGF-β1 in CFs were detected by RT-PCR and Western blot assay, respectively. Stimulation with chymase (15–30 ng/ml) for 3 h significantly upregulated TGF-β1 mRNA expression in a dose-dependent fashion (Fig. 2). The maximal mRNA level was approximately 2-fold at 30 ng/ml chymase (0.843 ± 0.031) compared with the untreated control (0.417 ± 0.021), although no significant effect was observed at 7.5 ng/ml (0.463 ± 0.035, $P > 0.05$). Meanwhile, incubation with chymase at the dose of 30 ng/ml for different periods (3–6 h) obviously increased TGF-β1 protein level in a time-dependent manner (Fig. 3), and the maximal value (1.167 ± 0.058) noted by 6 h was up to 2.5-fold compared with the control (0.487 ± 0.025). We also found that co-incubation with CHI (10 μM) significantly attenuated the TGF-β1 mRNA and protein expressions induced by 30 ng/ml chymase (0.553 ± 0.030 vs. 0.843 ± 0.031 and 0.713 ± 0.035 vs. 1.167 ± 0.058, respectively, $P < 0.01$).

To examine whether Ang II was involved in production of TGF-β1 caused by chymase, Ang II type-1 receptor (AT1) blocker valsartan and Ang II type-2 receptor (AT2) blocker PD123319 were incubated with CFs for 1 h prior to chymase. Our study showed that neither valsartan (10 μM) nor PD123319 (10 μM) had effects on the chymase-induced TGF-β1 mRNA and protein expressions (Figs. 2 and 3). These data demonstrate that chymase obviously upregulates TGF-β1 level independently of Ang II in CFs.

TGF-β1 is involved in chymase-induced CFs proliferation and collagen synthesis

As chymase increased mRNA and protein expressions of TGF-β1 in CFs, we thereby further explored whether TGF-β1 was implicated in the chymase-mediated cell proliferation and collagen synthesis using a specific TGF-β1 neutralizing antibody to block the effect of TGF-β1. As

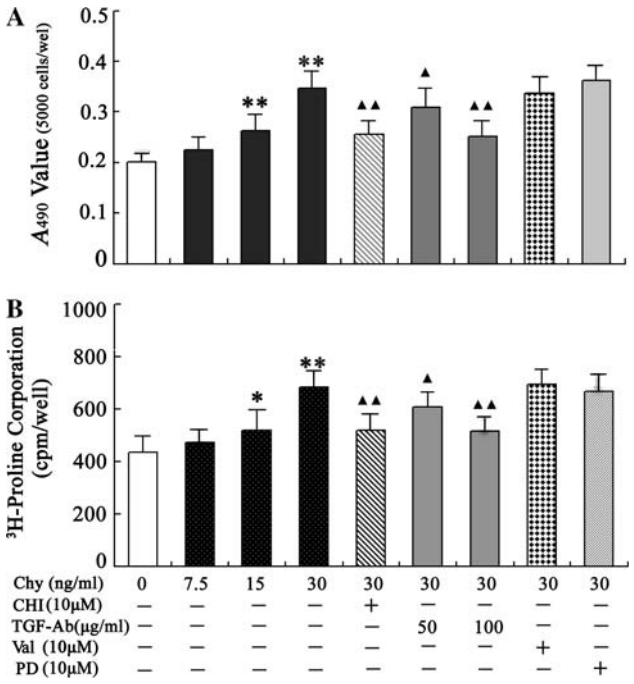

Fig. 1 Chymase promotes cell proliferation and collagen synthesis in a dose-dependent manner in rat CFs, which is reversed by TGF-β1 neutralizing antibody. CFs were serum-starved for 48 h, then stimulated with chymase (Chy) at indicated concentrations for 24 h. Some cells were pretreated with chymase inhibitor (CHI, 10 μM), TGF-β1 neutralizing antibody (TGF-Ab, 50, 100 μg/ml), valsartan (Val, 10 μM) or PD123319 (PD, 10 μM) for 1 h, and incubated for 24 h in the presence of 30 ng/ml Chy. Control cells only received vehicle. MTT assay (**A**) and ^{3}H -Proline incorporation (**B**) were employed to determine cell proliferation and collagen synthesis. Values are means ± SD and each bar represents six independent measurements. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group; $^{▲}P < 0.05$, $^{▲▲}P < 0.01$ versus the Chy (30 ng/ml) group

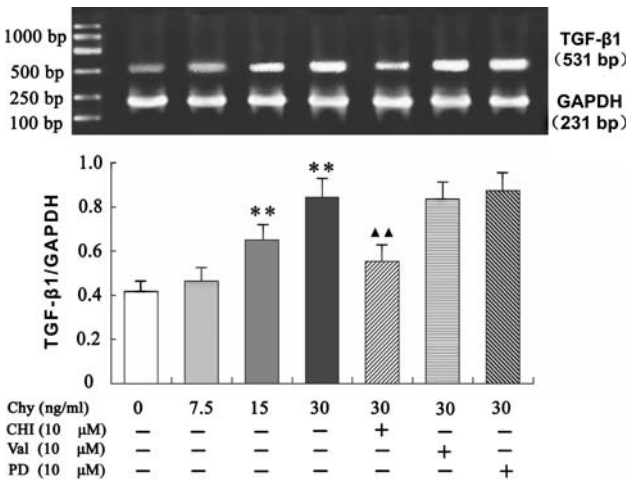

Fig. 2 Chymase induces TGF-β1 mRNA expression in a dose-dependent fashion independently of Ang II in rat CFs. The growth-quiescent CFs were treated with chymase (Chy) at the indicated concentrations for 3 h before harvest. Some cells were pretreated with chymase inhibitor (CHI, 10 μM), valsartan (Val, 10 μM) or PD123319 (PD, 10 μM) for 1 h followed by stimulation with 30 ng/ml Chy for 3 h. A representative image of electrophoresis of RT-PCR products in top panel and relative densitometric analysis expressed as ratio of TGF-β1/GAPDH in bottom panel are shown. Data are shown as means ± SD and represent three independent experiments. $^{**}P < 0.01$ vs. the control group; $^{▲▲}P < 0.01$ versus the Chy (30 ng/ml) group

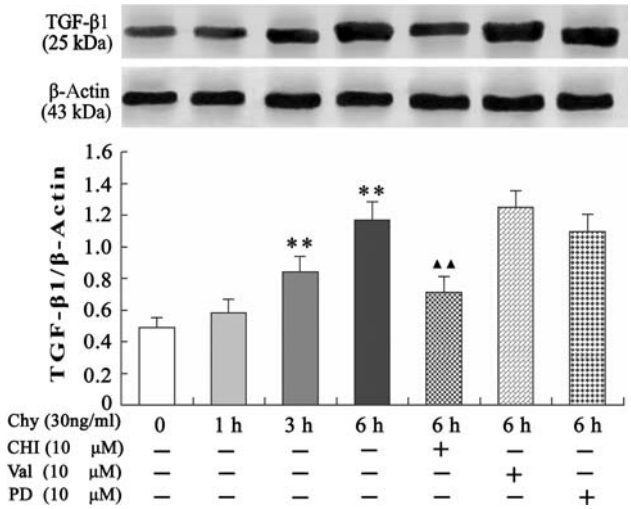

Fig. 3 Chymase induces TGF-β1 protein expression in a dose-dependent manner independently of Ang II in rat CFs. The synchronized cells were incubated with 30 ng/ml chymase (Chy) for the given periods before harvest. Some CFs were preincubated with chymase inhibitor (CHI, 10 μM), Val (10 μM) or PD (10 μM) for 1 h followed by addition with 30 ng/ml Chy for 6 h. A representative Western blot of TGF-β1 in top panel and relative densitometric analysis expressed as ratio of TGF-β1/ β-Actin in bottom panel are shown. Data are shown as means ± SD and represent three independent experiments. $^{**}P < 0.01$ versus the control group; $^{▲▲}P < 0.01$ versus the Chy (6 h) group

shown in Fig. 1A, B, chymase at 30 ng/ml markedly increased A_{490} Value and ^{3}H-Proline incorporation, which were significantly reversed by pretreatment with TGF-β1 neutralizing antibody (50, 100 μg/ml) in a dose-dependent manner. In contrast, these effects were not affected by pretreatment with valsartan (10 μM) and PD123319 (10 μM). Taken together, our results indicate that TGF-β1 is responsible for the chymase-mediated increases of CFs growth and collagen synthesis, which represents an autocrine action of CFs.

Chymase increases Smad2/3 phosphorylation in CFs

To determine the role of Smad proteins in chymase-induced cell proliferation and collagen synthesis, we observed the expressions of nuclear phosphorylated-Smad2/3 (P-Smad2/3) and cytoplasmic Smad2/3, Smad7 protein in CFs (Fig. 4). Western blot analysis showed that addition of 30 ng/ml chymase resulted in a strong increase of nuclear P-Smad2/3 peaking at 6 h (0.940 ± 0.046). After that, the activation of Smad2/3 was decreased mildly and remained higher level above the control (0.420 ± 0.036) by 24 h. In contrast, the level of Smad2/3 maintained constant in response to chymase during the whole observed time, indicating that chymase had no effect on Smad2/3 production. The expression of Smad7 protein was examined only at relatively low level (0.297 ± 0.042) in the cytosolic fraction in quiescent CFs, and was not significantly changed after chymase treatment. All these data suggest that chymase lead to activation of Smad signaling associated with P-Smad2/3 upregulation in CFs.

Chymase-induced Smad activation is TGF-β1 dependent

To address whether TGF-β1 or mitogen-activated protein kinase (MAPK) was implicated in chymase-induced Smad activation, we tested the effects of TGF-β1 neutralizing antibody, p38 MAPK inhibitor SB203580, and ERK inhibitor PD98059 on chymase-mediated expressions of Smad proteins by Western blot assay. As shown in Fig. 5, pretreatment with TGF-β1 antibody (100 μg/ml) markedly

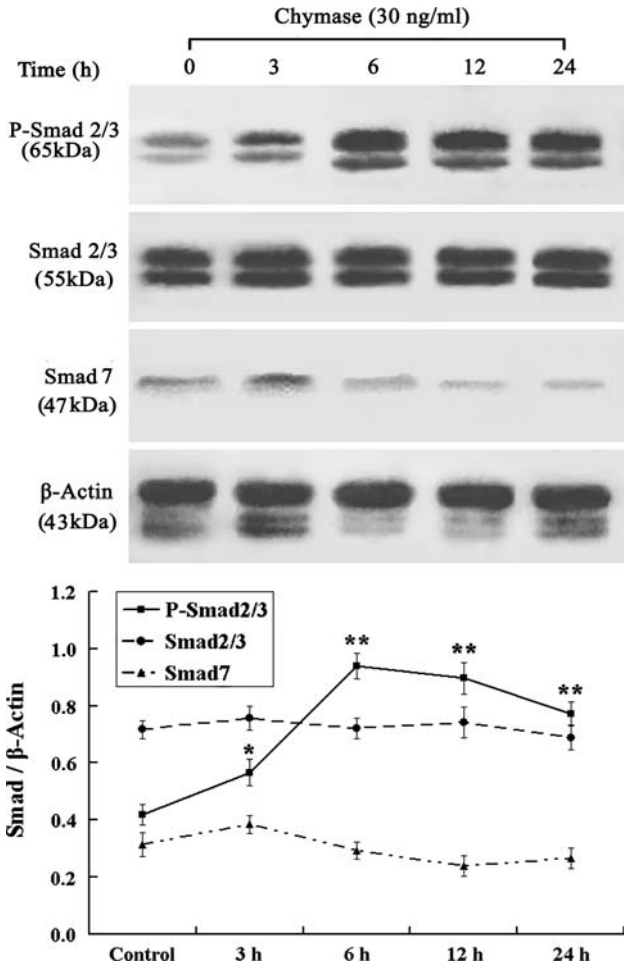

Fig. 4 Chymase induces upregulation of P-Smad2/3 in rat CFs. The growth-arrested cells were untreated or treated with 30 ng/ml chymase for the given periods and then protein levels were measured by Western blot. A representative Western blot of Smads in top panel and means ± SD data of three independent experiments in bottom panel are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group

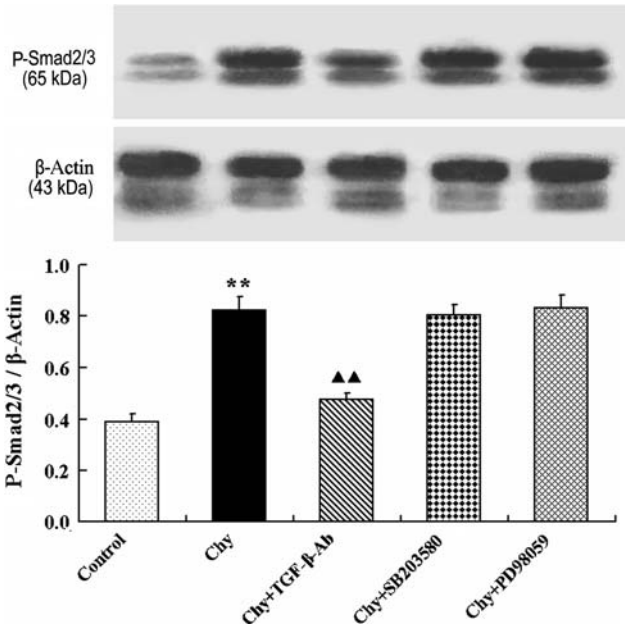

Fig. 5 Chymase-induced activation of Smad is mediated by TGF-β1, not by MAPK signaling. The growth-arrested CFs were treated with or without chymase (Chy, 30 ng/ml) for 12 h alone or preincubated with TGF-β1 neutralizing antibody (TGF-β-Ab, 100 μg/ml), SB203580 (20 μM), PD98059 (20 μM) for 1 h before 30 ng/ml Chy treatment. A representative Western blot of P-Smad2/3 in top panel and means ± SD data of three independent experiments in bottom panel are shown. $^{**}P < 0.01$ versus the control group; $^{▲▲}P < 0.01$ versus the Chy (30 ng/ml) group

diminished chymase-induced activation of Smad signaling by reduction of Smad2/3 phosphorylation, whereas SB203580 (20 μM) and PD98059 (20 μM) did not change the P-Smad2/3 level caused by chymase. These findings imply that chymase activates the Smad pathway through a TGF-β1-dependent mechanism rather than MAPK signaling.

Discussion

There are two main conclusions that can be drawn from this report. First, chymase promotes CFs proliferation and collagen synthesis through a TGF-β1-dependent mechanism. Second, chymase activates Smad pathway associated with increased P-Smad2/3 protein in CFs, which is mediated by TGF-β1, not by MAPK signaling. Therefore, our results raise the possibility that chymase might exert a key role on MF accompanied with increased cell proliferation and collagen synthesis via the TGF-β1/Smad signaling pathway.

A number of experimental results suggest that chymase plays an important role in the progression of cardiovascular diseases [9, 10, 13–15]. Even though increased activity of chymase has always been presumed to be associated with MF in vivo [10, 14], it has remained obscure whether this association is causal. In order to investigate a cause–effect relationship between chymase and MF, we characterized a direct effect of chymase on CFs of neonatal rats in vitro. Although, chymase has been reported to inhibit vascular smooth muscle cells growth [15], its effects on the proliferation of CFs and collagen metabolism are still unknown. Our work provides us first hand evidence that chymase can promote CFs proliferation and collagen synthesis, but the mechanism remains to be defined.

Considerable evidence displays that chymase not only induces Ang II generation by an "alternate" pathway [8–11] but also activate TGF-β1 from latent form [12]. Our study further showed that chymase led to dose- and time-dependent increase in TGF-β1 expression on both mRNA and protein levels of CFs, while the AT1 and AT2 blocker had no effect on the expression of TGF-β1 induced by chymase, indicating that Ang II is not participated in the production of TGF-β1 in rat chymase-treated CFs. This discovery is consistent with the previous study showing that rat chymase is able to generate and active TGF-β1, but not Ang II in vitro [12].

The functional roles of chymase-induced TGF-β1 expression in the CFs are subsequently analyzed. It is well-documented that TGF-β1 presents pleiotropic effects and regulates a diverse range of cellular responses including proliferation, apoptosis, differentiation, and collagen production. Particularly, the effect of TGF-β1 on proliferation is cell-type dependent [31]. It is interesting to note that TGF-β1 exerts completely different growth effects on CFs. Although there are reports suggesting TGF-β1 inhibits proliferation in CFs [32, 33], extensive evidence supports that TGF-β1 is able to stimulate CFs proliferation both in neonatal rat [19–21] and adult human [22]. These discrepant effects of TGF-β1 on cell proliferation may reflect not only species difference but also the cell state of differentiation and specific experimental conditions [34, 35]. Our results demonstrate that the CFs proliferation and collagen synthesis elicited by chymase are reversed by TGF-β1 neutralizing antibody, whereas, the AT1 and AT2 blocker do not alter these profibrotic effects in response to chymase. Accordingly, it can be concluded that TGF-β1 is responsible for the chymase-mediated increases of CFs growth and collagen synthesis independently of Ang II. Our study is similar to the study of Li et al. [21] showing that superoxide stimulates cell proliferation in neonatal rat CFs through upregulation of TGF-β1.

The intracellular signaling pathway that regulates chymase-induced profibrotic effects was also assessed in our in vitro model. In recent years, receptor-regulated Smads (R-Smads, including Smad2 and Smad3) and inhibitory-Smad7 (I-Smad7) have been well characterized as key downstream effectors of TGF-β signaling, and it is clear

that one of the initial steps of the activation of Smad pathway is the phosphorylation of R-Smads and Smad7 prevents R-Smads activation, thereby downregulates the TGF-β-signaling [24, 25]. Considering the pivotal role of chymase in the production of TGF-β1, we hypothesized that chymase might activate Smad signaling. Here, we observed that P-Smad2/3 in nuclear section was upregulated quickly after treatment with chymase, suggesting that activation of Smad2/3 might be one of the intracellular mechanism by which chymase mediated MF. In contrast to Smad2/3, we detected a weak expression of Smad7 protein in quiescent CFs, which was not markedly altered in chymase-treated CFs. However, Mori et al. [36] suggest that endogenous Smad7 is upregulated by TGF-β in normal skin fibroblasts. In fact, we also observed a slight upregulation of Smad7 at 3 h after chymase treatment, but the change was not significant compared to the control. This might indicate that Smad7 expression in neonatal rat CFs is very low and is not robustly induced by TGF-β1. More recently, increasing data provide novel evidence that Smad proteins are not exclusively activated by the classic TGF-β-triggered mechanism [25, 37], and there is a cross talk between Smad and MAPK signaling cascade [25, 38, 39]. Our results manifest that chymase-induced Smad activation is mediated by TGF-β1, not by MAPK signaling in rat CFs.

In conclusion, the present study provides us a novel insight into the role of chymase-dependent TGF-β1/Smad activation in vitro despite the relevance of this interaction needs verification in vivo in genetic models. In this regard, defining that production of TGF-β1 associated with Smad2/3 phosphorylation is essential for chymase-mediated fibrosis could contribute to the development of therapeutic strategies.

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