PAPEL DEL SISTEMA ANGIOTENSINA EN LA FISIOPATOLOGIA DE LA FIBROSIS PULMONAR

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2007

Departamento de Medicina

Facultad de Medicina

Programa de doctorado: Biopatología en Medicina

Bienio 2003-2005

PAPEL DEL SISTEMA ANGIOTENSINA EN LA FISIOPATOLOGIA DE LA FIBROSIS PULMONAR

Tesis presentada por María Molina Molina para optar al grado de doctor

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El trabajo realizado en esta tesis ha recibido financiación de los organismos oficiales: SEPAR, SOCAP, FUCAP y FIS-ISCIII

A “mi gente”, mi familia,
a los que están y a los que quedan en el recuerdo.
A Fuat, a mis padres y a mi hermano,
en honor a Pepin y en memoria de mi abuela

Agradecimientos

Me gustaría agradecer el resultado de esta tesis a todos los que han seguido a mi lado durante estos años y a los que han contribuido en su realización.

A mi hombre, por hacer sonreír mi corazón, llenarlo de fuerza, y ser la esencia y empuje de cada despertar, por ser mi alegría de vivir.

A mis padres, a los que adoro, por trasmitirme los valores humanos y enseñarme a luchar por lo que se sueña. Por todo lo que han pasado en la vida para conseguir algo mejor para los suyos.

A mi hermano, para que no deje el camino que le hace feliz, porque le quiero.

A mis abuelos, tíos y primos, especialmente a Pepin, por la unión familiar, donde residen mis vivencias más entrañables. A mi abuela Elin, un enorme ángel blanco, que dejó en mi alma una gran herencia.

A Xaubet, porqué además de excelente mentor es una buena persona y un tremendo profesional. Por formar un buen equipo.

Al servicio de neumología y a todos los miembros del equipo de investigación que han hecho posible este trabajo, técnicos y biólogos, por aunar esfuerzos. A Anna, por su ayuda y para que consiga su propósito. A Cesar Picado, Quim Mullol y Javi Pereda. Specially, I'd like to thanks Bruce Uhal for his ability to open my mind and to transmit his excitement for the science, an excellent professor and person. Also thanks to Elie, Nuria, Xiaopeng, Amal and Pampa for the good and unforgettable moments during my stay in Michigan State, US.

A todos los del zulo, Cristinita, Joan, Antonio, Tere, Maite, Ainoa, y M Carmen, porque en buena compañía el "background" es un pormenor.

A mis compañeros xalanistas y amigos-miembros de la "Unidad", en general por toda la ayuda y apoyo recibido durante años.

A Oriol, por ser un alma gemela, fuera y dentro, por esa forma de sentir y perseguir los sueños hasta vivirlos. A Elena, por existir y encontrarla de nuevo brillando. Y a los dos por compartir la magia del sur y los hermosos arándanos, hasta la luna.

A Ugeni, por estar siempre presente, por darme un calor especial y una ternura infinita, porque sentirlo cerca es impagable.

A Alfonso, por ser el ejemplo a seguir y el creador original de la "fresh fruit", entre otras muchas genialidades. A Cristina, por ser una superwoman en la manada. Y a los dos por formar una familia maravillosa.

A Carlos, por su mente privilegiada entre otras cualidades que lo hacen grande, para que siga con fuerza sus sueños, a pesar de las dificultades. A Pilar, por seguir fiel al amor y por ese cariño.

A Néstor, porque todo lo que ha conseguido se lo ha ganado con sudor y rabia, por ser además un padrazo y un enamorado de Julia, por ser único.

Y al resto de miembros, antiguos, presentes y futuros, para que, pase lo que pase, nunca se pierda la esencia de la Unidad.

A mis amigos/as que han seguido cerca a pesar del duro trabajo y el poco tiempo, que han hecho posible tantas veces un reset mental. A Isa y Sandra, por tanto vivido juntas en más de 20 años. A Susana, por ser la ternura en persona. A Carol, por ese incomparable “cómo”. A Jordi, Silvia, Hector y Lulu por compartir el olor de Vilanova.

A los que quiero y que todos estos años han propiciado esos buenos momentos que van más allá de la impresión de unas palabras en el papel. Gracias.

East Lansing, Septiembre 2006 *Barcelona, Enero 2007*

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Abreviaturas

AC: adenilato ciclasa

ANGEN: angiotensinógeno

ANG: angiotensina

AT1: receptor 1 de la angiotensina II

Ca^{++}: calcio iónico

Catep: catepsina

CMV: citomegalovirus

DAG: diacilglicerol

DLCO: capacidad de transferencia de monóxido de carbono

ECA: enzima convertora de angiotensina

EPID: enfermedad pulmonar intersticial difusa

ET-1: endotelina-1

FPI: fibrosis pulmonar idiopática

FVC: capacidad vital forzada

HGF: factor de crecimiento hepatocitario

JNK: c-Jun N-terminal quinasa

KGF: factor de crecimiento queratinocítico

MAPK: proteínas quinasa mitógeno-activado

MMPs/TIMPs: metaloproteinasas/inhibidores de metaloproteinasas

NAC: N-acetilcisteina

NF-KB: factor nuclear-KB

NII: neumonía intersticial idiopática

NINE: neumonía intersticial no específica

NIU: neumonía intersticial usual

PA: factor activador del plasminógeno

PK: proteina quinasa

PDGF: factor de crecimiento plaquetario

PGE-2: prostaglandina E2

PI: inositol fosfato

PLC: fosfolipasa C

RGE: reflujo gastroesofágico

SP: proteína del surfactante

TACAR: tomografía axial computerizada de alta resolución

TGF-β: factor transformador de crecimiento beta

TLC: capacidad pulmonar total

TNF-∝: factor de necrosis tumoral alfa

VEB: virus Ebstein Barr

1. Introducción

Fibrosis Pulmonar Idiopática: diagnóstico, evolución y tratamiento

La fibrosis pulmonar idiopática (FPI) es una enfermedad pulmonar intersticial difusa (EPID), progresiva y letal, con una supervivencia media de 3-4 años desde el diagnostico (1). Entre las neumonías intersticiales idiopáticas (NII), la FPI presenta un patrón histológico de neumonía intersticial usual (NIU) (2), y representa la entidad más frecuente y de peor pronóstico (3) *(Tabla 1)*.

Neumonías Intersticiales Idiopáticas
(Registro Español)

Fibrosis Pulmonar Idiopática	73.5%
N. Organizada Criptogenica	19.7%
N. Intersticial No Específica	3.3%
N. Intersticial Aguda	1.5%
Br. Respiratoria/EPID	1.2%
N. Intersticial Descamativa	0.8%
N. Intersticial Linfocítica	0

Tabla 1

A principios de este siglo el grupo multidisciplinario internacional de la American Thoracic Society (ATS) y la European Respiratory Society (ERS) redactó un consenso donde se compilan los criterios clínicos, radiológicos y anatomo-patológicos que permiten el diagnóstico de la FPI (4) *(Tabla 2)*.

La FPI afecta, predominantemente, a sujetos mayores de 50 años. Sus características clínicas son disnea y tos seca, que empeoran progresivamente, apareciendo en fases avanzadas insuficiencia respiratoria, hipertensión pulmonar y signos de cor pulmonale. A la auscultación respiratoria destacan los crepitantes bibasales. En un 50% de los casos se puede observar acropaquia en los dedos de las manos (4). La tomografía axial computerizada de alta resolución (TACAR) permite observar, en más de la mitad de los casos, imágenes características de NIU (5): afectación de predominio basal y periférico caracterizada por alteraciones reticulares, bronquiectasias de tracción, y áreas en panal subpleural, con escaso o ausente vidrio deslustrado, junto con disminución del volumen pulmonar. Actualmente se están realizando diferentes estudios con el objetivo de cuantificar la afectación tomográfica para permitir valorar con mayor precisión la progresión de la enfermedad.

La exploración funcional respiratoria objetiva una alteración ventilatoria restrictiva, con disminución de la capacidad vital forzada (FVC) y de la capacidad pulmonar total (TLC), junto con una disminución en la capacidad de transferencia de CO (DLCO). Cuando los pacientes no cumplen estrictamente los criterios clínico-radiológicos la biopsia pulmonar abierta sigue siendo necesaria, lo que ocurre en el 25-40% de casos. Los hallazgos histológicos principales en la FPI son daño epitelial persistente, acúmulo de fibroblastos/miofibroblastos, y abundante depósito de colágeno, con afectación predominante subpleural y en algunos casos discreta inflamación intersticial (6) *(figura 1)*. Se denomina cambios en panal a las zonas aéreas alargadas y quísticas con neumocitos tipo II anormales. La heterogeneidad temporal, zonas alteradas alternando con zonas de parénquima normal, es lo que la diferencia de otras patologías intersticiales, en especial la neumonía intersticial no específica (NINE) (4). La presencia de abundantes focos fibroblásticos-miofibroblásticos se correlaciona con la mortalidad en la FPI (2,7), y actualmente se intenta unificar los criterios de su cuantificación para su utilidad pronóstica (8).

Criterios diagnósticos de la FPI

Diagnostico clínico-radiológico: 4 mayores + 3 menores

Criterios mayores

- Exclusión de otras causas de EPID
- PFR: Alteración ventilatoria restrictiva y del intercambio de gases (↓ DLCO o ↑AaO2 en reposo o ejercicio)
- Alteraciones características en TACAR
- Hallazgos en biopsia transbronquial o en LBA que no sugieran otro diagnóstico

Criterios menores

- Edad > 50 años
- Disnea esfuerzo inicio insidioso, no explicada por otra causa
- Duración síntomas > 3 meses
- Estertores crepitantes bibasales persistentes

El hallazgo histológico de NIU requiere descartar otras enfermedades intersticiales que pueden asociar este patrón, como fibrosis asociada a colagenopatías, asbesto o amiodarona

Tabla 2

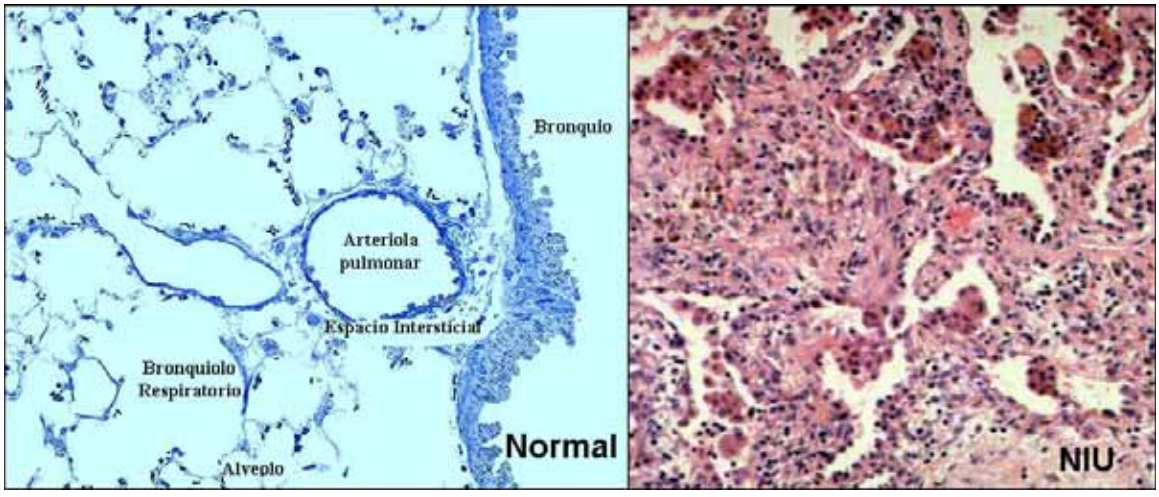


Figura 1

La evolución de la enfermedad es progresiva y cursa con episodios de agudización que empeoran la situación clínica del paciente. Los parámetros funcionales utilizados para evaluar el pronóstico de la FPI son la FVC y la DLCO, al diagnóstico y durante la evolución (9-11). Estudios recientes sugieren que la prueba de marcha de 6 minutos (12) y los cambios tomográficos (11) también pueden ser útiles para valorar la evolución de la FPI.

La incidencia estimada de la FPI es de 6-8 casos/100.000 habitantes/año (13). Actualmente, no existe un tratamiento que mejore el pronóstico ni modifique su progresión. La combinación farmacológica recomendada en las guías de consenso nacional e internacional, que consiste en la administración de glucocorticoides e inmunosupresores, ocasiona frecuentes efectos secundarios y no se ha demostrado que mejore la supervivencia de los pacientes (3-5). En este contexto, son múltiples los esfuerzos en el campo de la investigación biomédica con el objetivo de encontrar la terapia efectiva para esta enfermedad, para lo que es fundamental primero conocer los mecanismos fisiopatológicos que llevan al pulmón fibrótico *(figura 2)*.

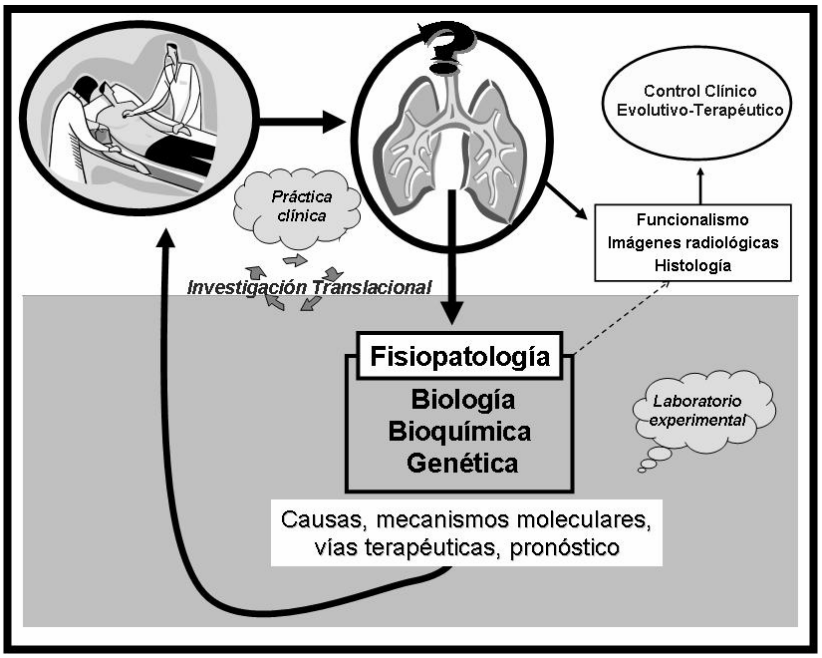


Figura 2

Factores genéticos:

Se ha observado que existe un 2% de agregación familiar en la FPI (14). Han sido varios los estudios realizados con el objetivo de investigar los genes alterados en esta enfermedad.

Se ha demostrado una mutación en el gen de la proteína C del surfactante pulmonar (SP-C), que lleva a la sustitución de leucina por glutamina, lo que provoca que la proteína no se pueda procesar correctamente y que la producción de surfactante pulmonar sea deficiente (15). Estos hallazgos sugieren que en la FPI familiar la alteración en el surfactante pulmonar puede contribuir en la fibrogénesis pulmonar (15). También se han asociado a la FPI y a su progresión los polimorfismos genéticos de algunas citoquinas, factores de crecimiento y enzimas, implicadas en la fibrogénesis pulmonar (16-20). Por lo tanto los polimorfismos que asocian un aumento en los factores pro-fibrogénicos pueden contribuir claramente en el desarrollo de la enfermedad.

Factores ambientales:

Diversos estudios han evaluado la posible implicación del virus Ebstein Barr (VEB) y el citomegalovirus (CMV) en la patogénesis de la FPI, ya que han sido detectados en forma latente en el pulmón de estos pacientes. Sin embargo, no existe evidencia suficiente para demostrar que los virus provoquen el desarrollo de la FPI (21-23). También se han estudiado otros factores de riesgo laborales y ambientales, sin encontrar una asociación causa-efecto (24, 25). El humo del tabaco es un factor de riesgo ambiental ampliamente estudiado en esta enfermedad, pero con resultados muy controvertidos (26,27). El reflujo gastroesofágico (RGE) ha sido otro elemento estudiado como posible causa, dado que se ha demostrado un aumento en la prevalencia de RGE en los pacientes con FPI (28). Sin embargo, al evaluar el efecto del tratamiento del RGE en la evolución clínica de la FPI, el escaso número de pacientes utilizado y la falta de controles con los que comparar, ha hecho imposible obtener conclusiones finales valorables (29).

A pesar de la hipótesis apoyada durante años en la que la FPI seria consecuencia de factores ambientales que actúan sobre sujetos predispuestos genéticamente, aún quedan sin determinar los factores exactamente implicados y responsables del proceso (6).

Tratamiento:

La pauta terapéutica estandarizada en el último consenso de los grupos ATS-ERS, combinación de glucocorticoides orales a dosis bajas junto con inmunosupresores (azatioprina o ciclofosfamida), no ha demostrado mejorar la supervivencia a lo largo de los años (3-5,27). Dada la ausencia de respuesta a los antiinflamatorios, durante las últimas décadas se han estudiado fármacos que actúan inhibiendo diferentes vías implicadas en el proceso fibrogénico *in vitro* y en modelos experimentales de fibrosis pulmonar, considerados "antifibróticos": antioxidantes, inhibidores del crecimiento celular de la matriz extracelular, así como de la apoptosis epitelial, y promotores del equilibrio entre las citoquinas y factores de crecimiento alterados (6).

Entre los fármacos considerados antifibróticos utilizados en ensayos clínicos experimentales en pacientes con FPI se encuentran interferon gamma-1b (IFN-γ1b), pirfenidona, bosentan, imatinib mesilato, etanercept, anticuerpos monoclonales humanos anti-TGF-β_1 y N-acetilcisteina (NAC). Hasta la actualidad ninguno de ellos, administrado aisladamente, ha demostrado mejorar la supervivencia de estos pacientes. El estudio que se realizó con NAC durante un año objetivó un menor deterioro en la DLCO y FVC (30), lo que, sumado a la ausencia de efectos secundarios, hace que este fármaco se esté añadiendo a la combinación terapéutica convencional.

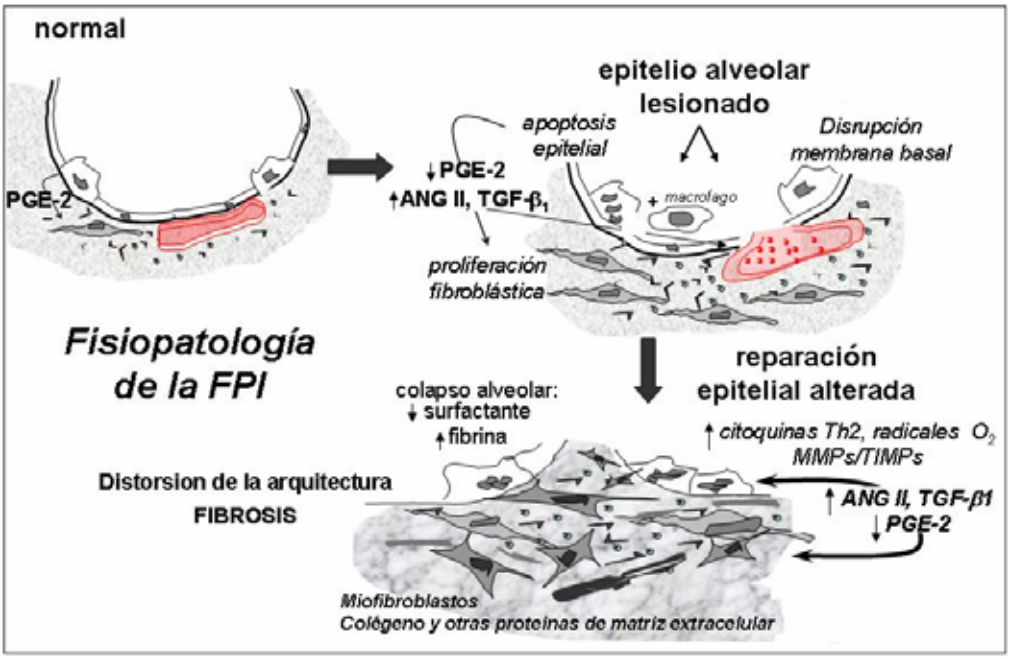
En la actualidad se están ampliando algunos de estos ensayos clínicos, dada la necesidad de una evaluación a largo plazo y con unos objetivos más específicos. Probablemente, la terapia efectiva pase por la combinación de varios de estos fármacos antifibróticos, actuando en los diversos factores implicados en el proceso fibrogénico pulmonar (31, 32). Asimismo, se ha sugerido que el recambio del epitelio alveolar dañado por células sanas de la

misma estirpe podría ser un tratamiento eficaz en la FPI. En este sentido, se evalúa el efecto de mitógenos de células epiteliales o células progenitoras capaces de diferenciarse a epiteliales alveolares. El estudio de la utilización de células troncales o células madre en el tratamiento de la FPI primero debe resolver diversos problemas básicos pero esenciales: número total de células necesarias, rechazo inmunológico, vía de administración adecuada, y el control de su diferenciación una vez llegado al tejido pulmonar (32), por lo que sigue siendo una opción a largo plazo.

Fisiopatología de la Fibrosis Pulmonar Idiopática

La fibrosis pulmonar resulta de la alteración en la reepitelización tras la lesión de las células alveolares (6,33), en la que existe un aumento en la apoptosis epitelial y en la síntesis de mediadores pro-fibróticos, lo que desencadena la proliferación de fibroblastos, la formación de focos de fibroblastos-miofibroblastos y el depósito incontrolado de matriz extracelular (*figura 3*). La progresión de este proceso patológico provoca disfunción de la unidad alveolo-capilar y alteraciones en la arquitectura pulmonar, que ocasionan insuficiencia respiratoria crónica y la muerte del paciente.

Figura 3



Este concepto de enfermedad epitelio-mesenquimal resulta de las evidencias observadas en múltiples estudios y sustituye al propuesto inicialmente, enfocado en la alteración inflamatoria alveolar como origen de todo el proceso fibrogénico (34), revocada ante la falta de respuesta a los tratamientos antiinflamatorios e inmunosupresores (4,6), así como ante la observación de la inducción de fibrosis sin previa inflamación en diferentes estudios experimentales. Sin embargo, el conocimiento sobre los mecanismos involucrados en la patogenia de la FPI resulta incompleto, por lo que sigue sin existir un tratamiento efectivo para la enfermedad (4,6,31,32)

Epitelio alveolar:

El alveolo es la unidad funcional pulmonar donde se realiza el intercambio de gases entre la circulación sanguínea y el aire atmosférico. El epitelio alveolar está formado por células epiteliales tipo I y tipo II que recubren toda la superficie (35,36). La membrana basal subepitelial separa este espacio del intersticio, localizado entre el epitelio alveolar y el endotelio capilar pulmonar, formado esencialmente por matriz extracelular y escasos fibroblastos (37).

Las células epiteliales tipo I ocupan el 90% de la superficie alveolar, están completamente diferenciadas, son elongadas y su morfología facilita el intercambio de gases. Estas células son metabólicamente activas y en su superficie albergan receptores para proteínas de la matriz extracelular, factores de crecimiento y citoquinas. Las células epiteliales tipo II son cuboidales, de núcleo redondeado, y contienen cuerpos lamelares citoplasmáticos donde almacenan el material para la síntesis de surfactante, sustancia que protege al alveolo del colapso al mantener la tensión superficial. Se localizan básicamente en las esquinas del epitelio alveolar y representan el 10% de la superficie celular. Estas células no están diferenciadas completamente, pueden funcionar como células madre al proliferar y diferenciarse hacia células epiteliales tipo I, realizando un papel fundamental al remplazar las células lesionadas y mantener la arquitectura normal del epitelio alveolar (35). Otras funciones atribuidas a las células epiteliales alveolares son la regulación de la actividad del citocromo P-450 monoxigenasa, el intercambio de iones y agua, y el metabolismo de la matriz extracelular (38,39).

Alteraciones en la FPI:

Cuando se dañan extensas áreas de epitelio alveolar existe una marcada disminución del surfactante, el alveolo se colapsa y disminuye la superficie alveolar, lo que contribuye a la restricción del volumen pulmonar (40). Se ha observado que en pacientes con FPI existen alteraciones en los componentes del surfactante pulmonar, como la disminución de la proteína A (SP-A) en el

lavado broncoalveolar (41). Asimismo, cuando la lesión epitelial es muy importante las células tipo II no sobreviven y, por lo tanto, pierden el potencial para reconstruir la superficie alveolar. La reepitelización normal del alveolo es crucial para mantener su homeostasis y evitar la fibrosis (6) (*figura 3*).

El epitelio alveolar normal se comporta como una barrera que evita la extravasación de proteínas endoteliales hacia la luz alveolar (42). En los pacientes con FPI existe extravasación de proteínas plasmáticas, fibrinógeno y otras sustancias al espacio alveolar (43,44). Diversos mediadores en la superficie de macrófagos y células epiteliales convierten el fibrinógeno en fibrina, lo que favorece la migración de los fibroblastos hacia el espacio alveolar (45,46) y la obliteración del alveolo al inducir el depósito de colágeno. En las células epiteliales normales los factores inhibidores de la proliferación y síntesis de colágeno, como la prostaglandina-E2 (PGE2), protegen de este efecto patológico (6). Los pacientes con FPI presentan una disminución del 50% en la cantidad de PGE2 del lavado broncoalveolar (47). Por lo tanto, la disfunción epitelial para generar PGE2 podría contribuir en la fibrogénesis pulmonar de la FPI.

Varios estudios demuestran la relevancia de la apoptosis epitelial acelerada en el desarrollo de fibrosis pulmonar. La inducción de apoptosis en las células epiteliales alveolares es suficiente para iniciar la respuesta fibrótica en diversos modelos animales (48) y la simple inhibición de dicha apoptosis previene la fibrosis inducida, tanto la proliferación de fibroblastos como el depósito de colágeno (49,50). En biopsias de pacientes con FPI se observa un aumento de la apoptosis epitelial principalmente en las zonas donde existe mayor concentración de miofibroblastos y colágeno (51). Asimismo, se ha relacionado la gravedad de la FPI con el incremento del ligando soluble del receptor Fas (asociado a la apoptosis) (52). Existen diferentes mediadores que regulan la apoptosis de las células alveolares epiteliales, como p53 y p21, que se encuentran alterados en tejido pulmonar de pacientes con FPI y en la fibrosis pulmonar inducida por bleomicina en animales (53,54). Estudios *in vitro* han demostrado que en cultivos de células epiteliales alveolares el estrés oxidativo

activa el c-Jun N-terminal quinasa (JNK), miembro de la familia de las proteínas quinasa mitógeno-activado (MAPK), lo que fosforila sus sustratos, c-Jun y el factor activador de trascripción 2 (55,56), e induce la síntesis de mediadores que contribuyen a la fibrogénesis pulmonar. En la FPI se ha observado un aumento de la actividad JNK y p38 MAPK en las células epiteliales, lo cual se acompaña de progresión en la fibrosis (57). Tras la lesión celular en la fibrogénesis pulmonar, los radicales libres de oxígeno y nitrógeno generados pueden inducir un aumento en la apoptosis epitelial (58,59). Estos radicales libres ejercen una acción directa sobre el DNA de la célula y además activan factores de trascripción, el factor nuclear-KB (NF-KB) y las MAPK (60), implicados en la muerte celular, proliferación y diferenciación, así como en la síntesis de citoquinas y factores de crecimiento profibróticos como angiotensina II (ANGII) y factor transformador de crecimiento beta (TGF-β1), por lo que se amplifica este proceso patológico.

La disregulación en la síntesis y secreción de factores de crecimiento tisular tiene un papel fundamental en la fibrogénesis pulmonar. Los factores de crecimiento tisular son un grupo de macromoléculas polipeptídicas que interactúan sobre receptores de membrana, originando una cascada de reacciones que regulan factores de trascripción y determinan la expresión génica. En la FPI las células epiteliales alteradas o apoptóticas secretan factores de crecimiento y citoquinas que favorecen la proliferación de fibroblastos y el depósito de matriz extracelular. Los más destacados son el TGF-β (61), la ANGII, el factor de crecimiento plaquetario (PDGF) (62), el factor de necrosis tumoral-α (TNF-α) (63) y la endotelina-1 (ET-1) (64). Por otro lado, el factor de crecimiento queratinocítico (KGF), un factor de proliferación y diferenciación de las células alveolares tipo II que promueve la reparación epitelial (65) y el factor de crecimiento hepatocitario (HGF) previenen la fibrosis pulmonar inducida por bleomicina en modelos animales murinos (66-68) y, como la PGE2 (47), se encuentran disminuidos en la FPI (69). Los diferentes efectos que provoca el desequilibrio entre factores que perpetúan el daño epitelial y aquellos que

favorecerían su correcta reparación en la fibrogénesis pulmonar apoyan la teoría que sugiere que el daño epitelial alveolar iniciaría la cascada fibrótica.
Finalmente, se ha postulado que la célula epitelial lesionada tiene además capacidad para trasformarse en miofibroblasto, lo que se denomina transición epitelio-mesenquimal. Esta hipótesis ha dado origen a varios estudios englobados dentro de la investigación sobre el origen de la formación de los miofibroblastos (70), lo que formaría parte esencial de la fisiopatología de la fibrosis pulmonar pero que, actualmente, sigue siendo una de las incógnitas a resolver.

Intersticio pulmonar:

El intersticio pulmonar normal se compone básicamente de proteínas de la matriz extracelular y escasos fibroblastos que confieren al pulmón las propiedades de distensibilidad y retracción elástica, además de mantener la homeostasis de la arquitectura pulmonar. La proliferación de fibroblastos y la síntesis de colágeno son una reacción fisiológica en el proceso reparativo de los tejidos dañados en cualquier órgano. Sin embargo, el depósito excesivo de colágeno y la disfunción de los fibroblastos alteran las funciones biológicas de órganos y tejidos.

Alteraciones en la FPI:
La proliferación incontrolada de fibroblastos, su diferenciación a miofibroblastos, así como la formación de focos de fibroblastos-miofibroblastos son características fundamentales de la FPI. Los miofibroblastos se caracterizan por expresar actina alfa del músculo liso (α-smooth muscle actin), proteína a la que se atribuye su capacidad de contracción. Estas células sintetizan y secretan factores profibróticos, además de provocar, junto con la excesiva formación de matriz extracelular, una alteración irreversible de la arquitectura del pulmón que confiere el carácter de “rigidez” pulmonar al dificultar su distensibilidad. En la matriz extracelular existe un excesiva producción y depósito de colágeno I, III, V, VI y VII, fibronectina, elastina y proteoglicanos (6) (*figura 3*). Diferentes estudios

sugieren que en estas alteraciones en la función de fibroblastos y de la matriz extracelular, así como en la apoptosis epitelial, tienen un papel fundamental la discontinuidad de la membrana basal epitelial y la acción de metaloproteasas (MMPs), factores de crecimiento y citoquinas de la respuesta inmune Th1/Th2 (71). Las metaloproteasas tienen como función principal mantener una correcta homeostasis en la formación de la matriz extracelular, pero también intervienen en la activación de diferentes factores de crecimiento, citoquinas, receptores y proteinasas. Existen diferentes factores de crecimiento implicados en la fibroproliferación y en la formación excesiva de matriz extracelular, siendo el TGF-β y la ANGII los de mayor relevancia. El TGF-β estimula la diferenciación y síntesis de colágeno, fibronectinas y proteoglicanos, así como la formación de miofibroblastos (70,72). La ANGII estimula la proliferación de fibroblastos y favorece el depósito de colágeno (73). Al contrario, el HGF y la PGE-2 inhiben la formación de miofibroblastos y el desarrollo de matriz extracelular (68, 74). La expresión de ambas moléculas se encuentra disminuida en los fibroblastos de pacientes con FPI (69, 74). El TGF-β_1 inhibe la producción de HGF, mientras que la expresión de PGE-2 favorece su activación (75). Por lo tanto, en la fibrogénesis pulmonar existe un desequilibrio entre los factores de crecimiento implicados en el proceso reparativo tisular a favor de aquellos que fomentan la proliferación fibroblástica, la formación de miofibroblastos y el depósito de colágeno.

Otro desequilibrio observado en la alteración mesenquimal de la FPI es la respuesta inmune Th1/Th2, en la que existe un aumento en la síntesis de IL-4, IL-5, IL-10 e IL-13 al activarse las células Th2 (76). En los modelos animales de fibrosis pulmonar donde se ha inhibido la respuesta Th1 se ha observado un predominio en los productos moleculares de la respuesta Th2 y mayor depósito de colágeno.

La producción excesiva de radicales libres de oxígeno y la disminución en su metabolización, provoca un aumento en la proliferación fibroblástica, en la

síntesis de factores de crecimiento y citoquinas que amplifican la respuesta fibrótica (59,60).

Sistema Angiotensina en la Fibrosis Pulmonar

Generalidades del Sistema Renina Angiotensina

El sistema renina-angiotensina (RAS) es conocido tradicionalmente como sistema endocrino que regula la homeostasis de fluidos, el metabolismo electrolítico y la presión arterial. La síntesis y secreción de renina, inducida al disminuir la perfusión renal y la concentración de sodio, escinde el angiotensinógeno (ANGEN), precursor sintetizado en el hígado, y se forma angiotensina I (ANG I). A través de la enzima convertora de angiotensina (ECA), localizada principalmente en el endotelio vascular pulmonar, la ANG I se convierte en angiotensina II (ANGII), octapéptido activo que es el principal efector del sistema (77). Este clásico concepto ha sido ampliado en las ultimas décadas tras relevantes observaciones (*figura 4*): a) Además de la ECA y la renina existen otras vías enzimáticas, catepsina D, catepsina G, catepsina E y quimasas, que intervienen en la síntesis de ANGII, b) Existen otros péptidos de la angiotensina, ANG III, ANG IV y angiotensina-(I-VII), con efectos biológicos poco conocidos, c) Se ha demostrado que, en diferentes órganos, los componentes de este sistema se pueden generar localmente (78) (*figura 4*).

De esta forma, se ha abierto un campo de investigación que evalúa el efecto de la ANGII como mediador local del crecimiento celular en diferentes tejidos.

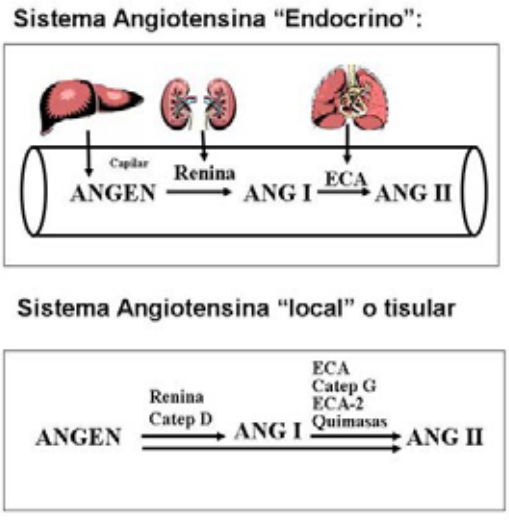


Figura 4

La ANGII actúa a través de los receptores 1 y 2 (AT1 y AT2), receptores proteicos G con siete dominios transmembrana (79). Los efectos de la ANGII al unirse al receptor AT1 han sido los más estudiados. Su acción sistémica provoca vasoconstricción arterial, aumento de la

presión arterial, activación del sistema nervioso simpático, liberación de vasopresina y aldosterona, y reabsorción de agua y sodio a través del riñón (80). Su acción autocrina y paracrina activa la proliferación de fibroblastos y formación de matriz extracelular (81, 82). Se ha demostrado que en el corazón induce hipertrofia ventricular (82) y, en la actualidad, se estudia su implicación como mediador de la fibrogénesis pulmonar en la FPI.

Sistema Angiotensina en la fisiopatología de la fibrosis pulmonar

Diferentes estudios han demostrado la existencia de un sistema angiotensina "local" en varios órganos y tejidos. La observación de concentraciones elevadas de ANGII en el intersticio cardiaco y ocular (50-500 pM), hasta 100 veces superior a la detectada en plasma (83,84), no es explicable por la difusión de ANGII desde el torrente sanguíneo. Además, los cultivos celulares derivados de diferentes órganos, exentos de componente vascular, expresan componentes del sistema angiotensina (85-87). Por lo tanto, existen evidencias suficientes para afirmar que los péptidos de la angiotensina pueden derivar del sistema "intrínseco" o local (independiente del endocrino) o "extrínseco" (desde los componentes externos mediante el sistema endocrino).

Angiotensinógeno

Las células epiteliales y los miofibroblastos pueden producir ANGEN *in vitro*. La inducción de apoptosis en células epiteliales alveolares incrementa la síntesis y secreción de ANGEN (88-90). Asimismo, se ha observado que los miofibroblastos aislados de pulmones fibróticos humanos y cultivados *in vitro* expresan ANGEN (91) (*figura 5*).

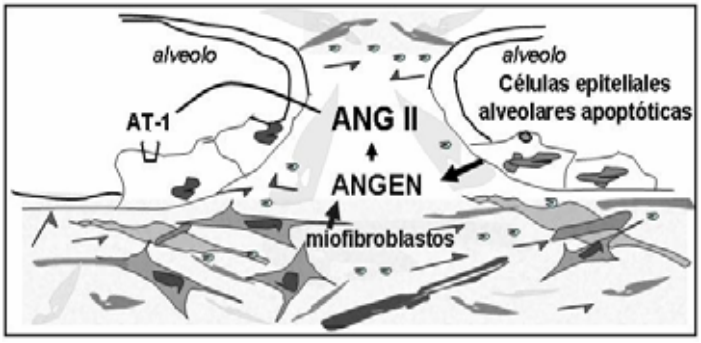


Figura 5

Además, la incubación del medio de cultivo de estos miofibroblastos con renina y ECA aumenta las concentraciones de ANGII, indicando que existe abundante ANGEN sintetizado constitutivamente que puede ser convertido a ANGII (91). Se ha demostrado que, en el modelo animal de fibrosis pulmonar inducida por bleomicina, el antagonismo específico del ANGEN inhibe el proceso fibrogénico (90). Estos datos demuestran que el ANGEN puede ser sintetizado localmente por el pulmón y que tiene una función importante en la fibrogénesis pulmonar.

Angiotensina II

Tanto en el suero como en el intersticio pulmonar, la vida media de la ANGII es corta (15s – 15 min). La unión con su receptor puede dar lugar a la internalización de la ANGII en diferentes células (87). Debido a ello, la interpretación de los niveles extracelulares es difícil.

En cultivos de células epiteliales se ha demostrado que la ANGII induce su apoptosis (91) y que este efecto es mediado por los receptores AT1. También se ha comprobado que la apoptosis epitelial inducida por Fas (88), TNF-α (89), amiodarona (92), o bleomicina (90) requiere la síntesis de novo de angiotensina ya que este efecto puede ser inhibido por oligonucleótidos anti-ANGEN, inhibidores de la ECA, y antagonistas del receptor AT1. Por otro lado, se ha observado que péptidos de la angiotensina derivados de los miofibroblastos pulmonares humanos son inductores de apoptosis epitelial (51,91). Estos datos sugieren que la ANGII es un activador de la apoptosis de células epiteliales alveolares que se observa en la fibrogénesis pulmonar.

La ANGII estimula *in vitro* la proliferación de fibroblastos fetales y humanos de forma directa mediante su unión al receptor AT1 y, por acción autocrina indirecta, al favorecer la síntesis de TGF-β (73). La ANGII también actúa sobre los fibroblastos al inducir la síntesis de colágeno mediante la activación del receptor AT1 (73). Otra forma indirecta mediante la cual la ANGII puede favorecer el depósito de colágeno es al alterar el balance del proceso fibrinolitíco/antifibrinolítico. La degradación de fibrina es un proceso mediado por el sistema de activación del plasminógeno (PA) (93) que transforma

plasminógeno en plasmina, enzima que degrada la fibrina. Estudios en modelos animales que sobreexpresan inhibidores de la activación del plasminógeno (PAIs) favorecen la fibrosis, mientras que la ausencia de PAIs disminuye la fibrogenesis (94). La ANGII puede inducir la síntesis del inhibidor del activador del plasminógeno-1 (PAI-1) (95), que al disminuir la plasmina inhibe la capacidad fibrinolítica y facilita el depósito de componentes de la matriz extracelular (94). Asimismo, en estudios cardiovasculares en modelos animales se ha observado que la ANGII aumenta la expresión del factor tisular, que convierte fibrinógeno en fibrina (95,96).

La ANGII puede regular la síntesis de otros factores de crecimiento implicados en la fibrosis pulmonar. Se ha demostrado que el HGF disminuye la fibrosis inducida por bleomicina en ratones (68). En estudios experimentales se ha observado que tanto la ANGII como el TGF-β son potentes inhibidores del HGF (97). Por otro lado, se ha descrito que la estimulación de fibroblastos con TGF-β incrementa la expresión génica de péptidos de la angiotensina (98,99). Asimismo, la estimulación de fibroblastos pulmonares normales con ANGII aumenta la expresión y la actividad proteica del TGF-β1 (99). Por lo tanto, es posible que ambos mediadores pro-fibróticos se sobreregulen respectivamente.

<u>Enzimas y proteasas del sistema angiotensina</u>

En el pulmón no se encuentra renina, pero se ha encontrado catepsina D, una proteasa que puede realizar la conversión de ANGEN a ANG I (100) *(figura 4)*. La catepsina D es una proteasa lisosomal que se activa tras la inducción de fibrosis con bleomicina en células epiteliales alveolares murinas y provoca un aumento de su apoptosis al favorecer la síntesis de péptidos de angiotensina (101). La catepsina D se encuentra incrementada en la fibrosis pulmonar inducida por bleomicina (101) y en pulmones humanos fibróticos (102). También existen otras proteasas que podrían influir en el proceso de síntesis de ANGII como la catepsina E (103), pero de las que se desconoce su implicación en la fibrogénesis pulmonar.

La enzima más estudiada en la conversión de ANG I a ANGII es la ECA. Existen dos isoformas, una ligada a membrana y la otra soluble. La ligada a la membrana se une mediante una secuencia corta hidrofóbica C-terminal a la membrana plasmática y se encuentra en la mayoría de los órganos. Se expresa en el endotelio vascular de la circulación pulmonar y es la principal responsable de la conversión de ANG I a ANGII en la circulación (104). La ECA soluble se encuentra en la mayoría de los fluidos corporales (105). Los cultivos primarios de células epiteliales alveolares expresan RNAm de ECA (91). Por otro lado, se ha observado que la ECA está sobreexpresada en los pulmones fibróticos de animales y humanos (106,107). En un estudio en el que se evaluó la inserción/delección del alelo D (polimorfismo I/D) de la ECA en pacientes con fibrosis pulmonar moderada-severa, el alelo D confería mayor cantidad de ECA comparado con el alelo I, y presentaban un aumento en su expresión un 15% mayor que en la población general (19). A pesar del escaso numero de casos estudiados, estos hallazgos indican que los polimorfismos de la ECA influyen en su síntesis, lo que podría conferir predisposición a desarrollar fibrosis pulmonar. En modelos animales de fibrosis pulmonar y en cultivos *in vitro* la inhibición de la ECA mediante captopril o enalapril disminuye el proceso fibrogénico pulmonar (49,108,109).

Se han encontrado otras enzimas diferentes a la ECA que también convierten ANGI en ANGII entre las que se incluyen quimasas, catepsina G y ECA-2, enzimas descritas recientemente en diversos estudios cardiovasculares (103).

Receptores de la ANGII

En el pulmón humano normal el receptor AT1 se localiza en las células musculares vasculares, macrófagos y en el estroma peribronquial (110). El receptor AT2 se expresa en el epitelio bronquial, glándulas submucosas y células endoteliales (110).

En modelos murinos de fibrosis pulmonar, la administración de bleomicina aumenta significativamente la expresión de AT1 (111), induce apoptosis epitelial

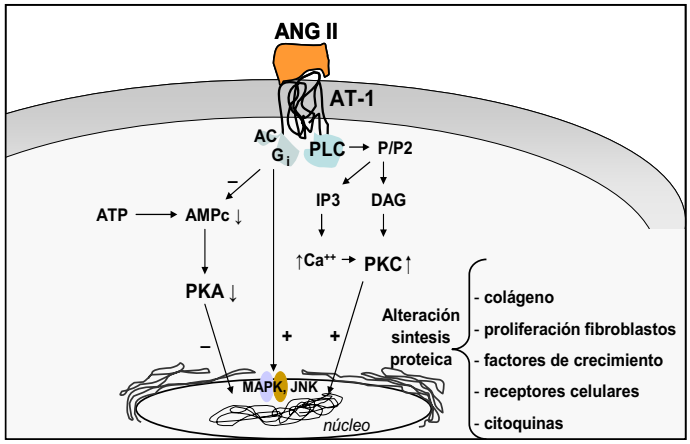
y fibrosis pulmonar, mientras que su antagonismo, mediante losartan o candesartan, disminuye estos efectos (111-112).

Señales intracelulares mediadas por la unión ANG II - AT1:

La unión de ANGII al receptor AT1 desencadena una cascada de señales intracelulares que finaliza en la síntesis de productos intermediarios, efectores de la acción de la ANGII.

La activación de la fosfolipasa C-beta (PLC-β) hidroliza el fosfatidilinositol-4,5-bifosfato (PIP2) para generar inositol-1,4,5-trifosfato (IP3) y diacilglicerol (DAG). Se produce un incremento del Ca^{++} intracelular a través de los canales de calcio, bajo la influencia de la proteína G y por el aumento del IP3 a través del retículo endoplasmatico.

Figura 6



Este incremento del calcio junto con el DAG induce la accion fosforilativa de la proteína quinasa C (PKC) y otros miembros de la familia de serina y tirosina quinasas (113), que ejercen funciones mitogénicas e inducen la proliferación celular (*figura 6*).

Por otro lado, la unión ANGII - AT1 puede inducir la activación de la proteína G inhibidora (Gi) (114), que inhibe la actividad adenilato ciclasa y disminuye el nivel intracelular de AMPcíclico (AMPc). Niveles disminuidos de AMPc inhiben la actividad de la protein quinasa A (PKA), lo cual disminuye la capacidad de inhibición de la fibroproliferación y depósito de colágeno, características que hacen que el AMPc sea considerado antifibrótico (115) *(figura 6)*.

En las células renales, la unión ANGII - AT1 también interviene en la activación de la fosfolipasa A2, que convierte la fosfatidilcolina en ácido araquidonico (116).

El ácido araquidónico se metaboliza a prostaglandinas y tromboxano A2 mediante la ciclooxigenasa, y a ácidos hidroxieicosatetraenoicos y leucotrienos a través de la lipoxigenasa.

Se ha observado que la unión ANGII – AT1 estimula la regulación de la expresión genética de proteínas quinasas intracelulares y factores de trascripción nuclear que activan gran número de factores de crecimiento y de proteínas de matriz extracelular y favorecen el crecimiento celular (117) *(figura 6)*. Además, la ANGII unida al AT1 puede ser endocitada y unirse directamente a los receptores con DNA asociado, regulando la trascripción genética de renina, ANGEN y PDGF (103).

Modelo experimental de fibrosis pulmonar inducida por bleomicina en rata

La base para la utilización de modelos animales en la investigación de los mecanismos fibrogénicos son las similitudes estructurales, bioquímicas y moleculares entre la reacción fibrótica en humanos y animales. La experimentación en animales es el único procedimiento que permite valorar en tiempo real el efecto de las interacciones genéticas, bioquímicas y medioambientales que provocan fibrosis pulmonar y los mecanismos fisiopatológicos implicados. La utilidad y elección del modelo animal a utilizar depende de las características del evento fibrótico a estudiar, las hipótesis y objetivos de la investigación, así como de la logística de la que se dispone.

Los animales de experimentación más utilizados son el ratón y la rata, por su fácil manipulación y el menor coste. Entre los agentes fibrogénicos, el más utilizado es la bleomicina, un complejo glicoproteico anti-neoplásico (118) que actúa mediante un efecto oxidante, escindiendo el DNA y aumentando los mediadores fibrogénicos (119,120). Los efectos citotóxicos de la bleomicina se producen básicamente en la piel y los pulmones (121), donde alcanza las mayores concentraciones tras su administración parenteral, al tener menor actividad de la hidrolasa celular que inactiva el fármaco. La vía de administración más común es la intratraqueal, en dosis única, entre 2.5 y 7.5 mg/kg (121), con lo que se provocan áreas parcheadas en localización peribronquial histológicamente similares a la FPI (122). Las lesiones inducidas por este agente incluyen inflamación, lesión epitelial con hiperplasia reactiva y apoptosis, alteración de la membrana basal, fibrosis de distribución heterogenea (intra-alveolar, subpleural o bronquiolocéntrica), así como focos de células mesenquimales en forma de huso de apariencia similar a los focos de miofibroblastos (123). El modelo de bleomicina ha sido utilizado para: a) estudiar los mecanismos de acción de factores de crecimiento (TGF-β, ANGII y ET-1) y de diversas citoquinas, así como el efecto terapéutico de su inhibición, b) valorar la modulación transgénica en la respuesta fibrótica e identificar posibles *locus*

reguladores en la predisposición genética a la fibrosis pulmonar, c) estudiar la alteración celular epitelial, fibroblástica y de la matriz extracelular (124).

No existe ningún modelo animal de fibrosis pulmonar que mimetice con exactitud la progresión y cronicidad de la FPI (125). Aunque el modelo de bleomicina instilada es fácilmente reproducible, cabe recordar que es un modelo de lesión de rápida instauración y duración determinada. El desarrollo del daño pulmonar por bleomicina en los diferentes modelos animales es bastante similar. La progresión se caracteriza por una fase inflamatoria aguda (1-3 días post-instilación intratraqueal, días mas tarde en la parenteral), donde se pueden estudiar diferentes variables de inflamación y edema, y una fase subaguda (4-21 días post-instilación intratraqueal), caracterizada por la síntesis de tejido conectivo (121). A partir del día 21 se considera que existe la fase de reepitelización, cuando el tejido conectivo se metaboliza.

El modelo de fibrosis pulmonar inducida por bleomicina intratraqueal en rata es el modelo que se ha utilizado en el presente trabajo para estudiar el efecto del antagonismo del receptor AT1 mediante losartan sobre la fibrogénesis pulmonar y los cambios moleculares en la PGE2 que podrían contribuir en su acción antifibrótica *(figura 7)*.

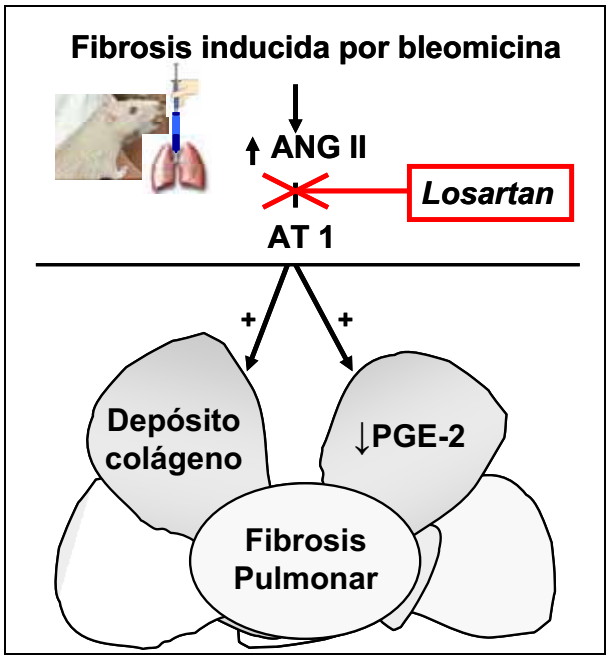


Figura 7

Hipótesis de trabajo

a) La angiotensina II ejerce un efecto relevante en la fibrogénesis pulmonar, aumenta el depósito de colágeno y contribuye al desequilibrio de los factores de crecimiento celular implicados.
b) Este efecto se debe básicamente a su unión con el receptor 1 (AT1).
c) El antagonismo del receptor 1 de la angiotensina II, mediante losartan, inhibe la fibrogénesis pulmonar.
d) La prostaglandina E2 es una molécula que inhibe la fibrosis pulmonar al disminuir el depósito de colágeno.
e) El antagonismo del receptor 1 de la angiotensina II inhibe la disminución de la prostaglandina E2 que se produce en la fisiopatología de la fibrosis pulmonar.
f) En la FPI existe un aumento en la síntesis de los péptidos de angiotensina.

Objetivos

a) Estudiar el papel de los péptidos de la angiotensina en la fibrogénesis pulmonar y su impacto en la FPI.
c) Estudiar el efecto del antagonismo del receptor AT1, mediante losartan, en la fibrosis pulmonar experimental.
d) Evaluar la posible acción reguladora del antagonismo del receptor AT1 sobre otros mediadores cuya alteración se encuentra implicada en la FPI: TGF-β1 y PGE2.
b) Investigar qué células sintetizan los péptidos de la angiotensina en pulmones afectos de FPI.

2. Artículos publicados

1º: Losartan attenuates bleomycin induced lung fibrosis by increasing prostaglandin-E2 synthesis

2º: Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis

3º: Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages

Losartan attenuates bleomycin induced lung fibrosis by increasing prostaglandin E 2 synthesis

M Molina-Molina, A Serrano-Mollar, O Bulbena, L Fernandez-Zabalegui, D Closa, A Marin-Arguedas, A Torrego, J Mullol, C Picado and A Xaubet

Thorax 2006;61;604-610; originally published online 6 Apr 2006;
doi:10.1136/thx.2005.051946

INTERSTITIAL LUNG DISEASE

Losartan attenuates bleomycin induced lung fibrosis by increasing prostaglandin E_2 synthesis

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Thorax 2006;**61**:604–610. doi: 10.1136/thx.2005.051946

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Received 21 August 2005
Accepted 27 February 2006
Published Online First 6 April 2006

Background: The angiotensin system has a role in the pathogenesis of pulmonary fibrosis. This study examines the antifibrotic effect of losartan, an angiotensin II type 1 receptor antagonist, in bleomycin induced lung fibrosis and its possible implication in the regulation of prostaglandin E_2 (PGE_2) synthesis and cyclooxygenase-2 (COX-2) expression.

Methods: Rats were given a single intratracheal instillation of bleomycin (2.5 U/kg). Losartan (50 mg/kg/day) was administrated orally starting one day before induction of lung fibrosis and continuing to the conclusion of each experiment.

Results: Losartan reduced the inflammation induced by bleomycin, as indicated by lower myeloperoxidase activity and protein content in the bronchoalveolar lavage fluid. Collagen deposition induced by bleomycin was inhibited by losartan, as shown by a reduction in the hydroxyproline content and the amelioration of morphological changes. PGE_2 levels were lower in fibrotic lungs than in normal lungs. Losartan significantly increased PGE_2 levels at both 3 and 15 days. A reduction in COX-2 expression by bleomycin was seen at 3 days which was relieved by losartan.

Conclusions: The antifibrotic effect of losartan appears to be mediated by its ability to stimulate the production of PGE_2. Losartan, which is already widely used clinically, could be assessed as a new treatment in lung fibrosis.

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic interstitial lung disease which is associated with the histological appearance of usual interstitial pneumonia. The poor prognosis of IPF patients, with a mean survival period of 2–4 years, and the inefficacy of current treatment based on corticosteroids and immunosuppressive drugs, underlines the need for new therapeutic strategies.[1] IPF is characterised by the loss of lung architecture through increased epithelial cell apoptosis and abnormal wound repair, leading to the formation of fibroblast-myofibroblast foci and extracellular matrix deposition.[2–4] This pathological process is related to the interactions of several cytokines, chemical mediators, and growth factors derived from epithelial and mesenchymal cells.[5 6] Experimental lung fibrosis induced by bleomycin is a well studied model of fibrogenesis supported by ample literature. This model of pulmonary fibrosis resembles that seen in humans and has been used to assess the effects of potential therapeutic agents.

Angiotensin II (ANGII) is produced by proteolytic cleavage of its precursor angiotensin I by angiotensin converting enzyme (ACE). Experimental evidence suggests that ANGII regulates the fibrotic response to tissue injury. It has been reported that ANGII plays an important role in cardiac, renal, hepatic, and pancreatic fibrogenesis.[7–10] It has also been shown that ANGII is a crucial mediator in the pathogenesis of pulmonary fibrosis. ANGII induces proliferation of human lung fibroblasts and production of lung procollagen via activation of angiotensin type 1 receptor (AT1).[11–13]

It is known that losartan, a selective AT1 receptor antagonist, inhibits the proliferation of human lung fibrotic fibroblasts induced by ANGII in vitro.[12 13] Moreover, losartan inhibits the deposition of lung collagen in the rat model of bleomycin induced pulmonary fibrosis.[11]

The mechanisms involved in the pulmonary antifibrotic effect of losartan have been widely investigated but are still not fully understood. One potential mechanism is the regulation of other inflammation mediators and growth factors. There is evidence that the antifibrotic effect of losartan is mediated through AT1 receptors and involves at least the downregulation of transforming growth factor β (TGF-β), a profibrotic mediator.[11]

It has been reported that prostaglandin E_2 (PGE_2) is a potent inhibitor of fibroblast proliferation, collagen synthesis, and fibroblast to myofibroblast differentiation.[4] Bronchoalveolar lavage (BAL) fluid from patients with IPF has been found to contain 50% less PGE_2 than normal individuals.[14] Furthermore, alveolar macrophages and fibroblasts have a reduced capacity to produce PGE_2 in vitro.[15 16] The failure to synthesise PGE_2 has been shown to be associated with a decreased capacity to upregulate cyclooxygenase 2 (COX-2).[16–18]

The main objective of this study was to investigate the antifibrotic effect of losartan and its possible implication in the regulation of PGE_2 synthesis and COX-2 expression in a rat model of bleomycin induced pulmonary fibrosis. Some of the results of this study have previously been reported in the form of abstracts.[19 20]

METHODS

Animals

Adult male pathogen free Sprague-Dawley rats weighing 225–250 g at the beginning of the studies were obtained from Criffa (Iffa Credo, France). The animals were maintained in a controlled environment and fed on rodent chow (A04; Panlab, Barcelona, Spain) and tap water ad libitum.

Abbreviations: ACE, angiotensin converting enzyme; ANGII, angiotensin II; AT1, angiotensin type 1 receptor; COX-2, cyclooxygenase-2; HP, hydroxyproline; IPF, idiopathic pulmonary fibrosis; MPO, myeloperoxidase; PGE_2, prostaglandin E_2; TGF-β, transforming growth factor β

Chemicals

Bleomycin sulphate was obtained from Almirall-Prodesfarma (Barcelona, Spain), halothane (Fluothane) from Zeneca Farma (Pontevedra, Spain), and sodium pentobarbital from Normon (Madrid, Spain). Losartan was provided by Merk and Co (West Point, PA, USA).

Experimental model

Animals were anaesthetised under halothane and a single dose of 2.5 U/kg bleomycin dissolved in sterile saline (0.9% NaCl) was instilled intratracheally via the transoral route by a small glass device developed especially for it.[21] Control animals received the same volume of intratracheal saline solution. The dose of bleomycin was selected from previous experiments to cause no mortality but consistent biochemical and histological damage.[21 22] The duration of each experiment was 3 or 15 days post-instillation. The animals were sacrificed at the end of each experiment by a lethal injection of sodium pentobarbital (100 mg/kg ip) followed by exsanguination from the abdominal aorta. Lung tissues were weighed and processed separately for bronchoalveolar lavage, biochemical, and histological studies as described below.

Experimental groups

Animals were randomly distributed into four groups in each experiment: (1) sterile saline solution (vehicle for bleomycin) + water (vehicle for losartan) (n = 4); (2) sterile saline solution + losartan (n = 4); (3) bleomycin + water (n = 8); (4) bleomycin + losartan (n = 8).

Losartan (50 mg/kg/day) or water was administered orally (at 09.00 hours) starting one day before the instillation of bleomycin and continuing up to the end of the experiment. The treatment was dissolved in a final volume of 1–2 ml distilled water. The experimental groups were repeated twice at 3 days and at 15 days. Oral administration was selected as this is usual in the clinical setting, and the dose level and schedule were based on previous studies.

Biochemical studies

Neutrophilic infiltration was assessed by measuring myeloperoxidase (MPO) activity.[23] MPO was measured photometrically with 3,3′,5,5′-tetramethylbenzidine as a substrate. Lung samples were macerated with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer at pH 6.0. Lung homogenates were disrupted for 30 seconds using a Labsonic sonicator (B Braun) at 20% power and submitted to three cycles of snap freezing in dry ice and thawing before a final 30 second sonication. Samples were incubated at 60°C for 2 hours and then spun down at 4000*g* for 12 minutes. The supernatants were collected for MPO assay. Enzyme activity was assessed photometrically at 630 nm. The assay mixture consisted of 20 µl supernatant, 10 µl tetramethylbenzidine (final concentration 1.6 mM) dissolved in DMSO, and 70 µl H_2O_2 (final concentration 3.0 mM) diluted in 80 mM phosphate buffer, pH 5.4. The results are expressed as units (U) MPO activity per lung.

Lung hydroxyproline (HP) content was measured as an indicator of collagen deposition by the method outlined by Woessner.[24] Lung samples were homogenised and then hydrolysed in 6 N HCl for 18 hoursat 110°C. The hydrolysate was neutralised with 2.5 M NaOH. Aliquots (2 ml) were analysed for HP content after the addition of chloramine T (1 ml), perchloric acid (1 ml), and dimethylaminobenzaldehyde (1 ml). Samples were read for absorbance at 550 nm in a spectrophotometer. The results are expressed as µg HP per lung.

Bronchoalveolar lavage fluid

BAL fluid was obtained by cannulating the trachea and lavaging four times with 10 ml NaCl 0.9%. The BAL fluid was centrifuged (300*g*, 10 minutes, 4°C) and the supernatant was used for biochemical studies. Total protein concentration was measured by a standard dye technique (BioRad; Munich, Germany). The levels of PGE_2 were measured by a competitive enzyme immunoassay kit (Cayman Chemical; Ann Arbor, MI, USA) following the manufacturer's protocol.

RNA preparation and RT-PCR of COX-2

Total RNA was prepared with Trizol reagent following the manufacturer's instructions (GibcoBRL, Life Technologies) and RNA concentrations were calculated from A_{260} determinations. RNA integrity and loading amounts were assessed by examining 18S and 28S ribosomal RNA banding of samples electrophoresed in 1% agarose gel under non-denaturing conditions and stained with ethidium bromide. Analysis of COX-2 and β-actin mRNA expression was done using a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method. One µg total RNA was used and the sequences amplified by the Life Technologies One Step RT-PCR System according to the manufacturer's protocol. The forward primer for COX-2 was 5′-GCT-GTA-CAA-GCA-GTG-GCA-AA-3′ and the reverse primer was 5′-ATG-GTG-GCT-GTC-TTG-GTA-GG-3′. For β-actin the forward primer was 5′-TCA-TGA-AGT-GTG-ACG-TTG-ACA-TCC-GT-3′ and the reverse primer was 5′-CCT-AGA-AGC-ATT-TGC-GGT-GCA-CGA-TG-3′. Sequences were resolved by electrophoresis in denaturing 1.8% agarose gel and staining with ethidium bromide.

Lung histological studies

Histological evaluation was carried out on lungs that were not lavaged. The lungs were first perfused through their main bronchus with a fixative solution (10% neutral buffered

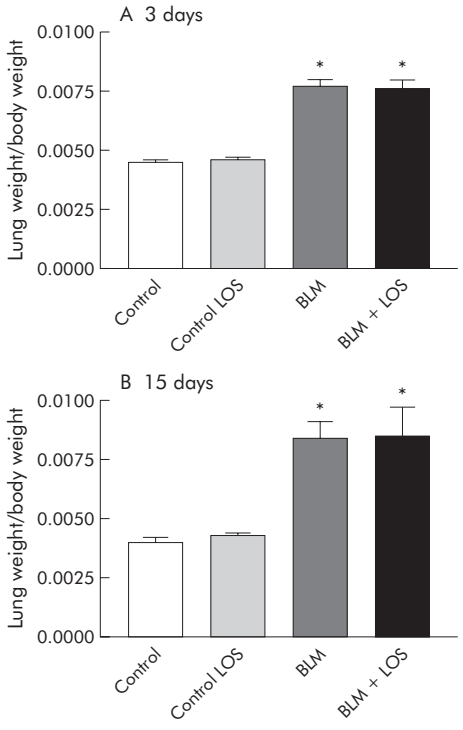

Figure 1 Relationship between lung weight and body weight at (A) 3 days and (B) 15 days. There was a significant increase in the lung/body weight relationship after bleomycin (BLM) administration at 3 days (*p = 0.001 versus control group) and at 15 days (*p<0.001 versus control group). Losartan (LOS) had no effect on this relationship (3 days: p = 0.84; 15 days: p = 0.88). Statistical analysis was performed by one way ANOVA followed by Student's *t* test.

formalin) at a pressure of 25 cm H_2O, immersed in the fixative for 12–24 hours, and blocks were taken. Tissue paraffined blocks were cut into 4 μm thick sections and stained with haematoxylin-eosin and Masson's trichrome to identify inflammatory cells, connective tissue, and collagen deposition. The histological changes were evaluated by a descriptive method.

Statistical analysis

Data are expressed as mean (SE) with 95% confidence intervals (CI) of n experiments. Statistical analysis was carried out by analysis of variance (ANOVA) followed, when differences were significant, by appropriate post hoc tests including the Newman-Keuls tests. Differences between groups were tested using the paired Student's *t* test (GraphPad Software Inc, San Diego, CA, USA). Differences were considered statistically significant when p was <0.05.

RESULTS

Effect of losartan on bleomycin induced lung inflammation and collagen deposition

The mean (SE) lung/body weight ratio was significantly increased in fibrotic rats induced by bleomycin both at 3 days (control group: 0.0045 (SE 0.0001) (95% CI 0.0046 to 0.0047); bleomycin group: 0.0077 (SE 0.0003) (95% CI 0.0071 to 0.0083)) and at 15 days (control group: 0.004 (SE 0.0002) (95% CI 0.0037 to 0.0043); bleomycin group: 0.0084 (SE 0.0012) (95% CI 0.0071 to 0.0098)). Losartan treatment did not have a significant effect on this ratio (fig 1A and B).

An increase in MPO activity was observed in bleomycin exposed rats compared with the control groups at 3 and 15 days (fig 2A and B) after bleomycin instillation (3 days: control group 146.8 (5.2) U/lung (95% CI 138.4 to 155.1), bleomycin group 399 (28.7) U/lung (95% CI 350.3 to 447.7); 15 days: control group 279.5 (103.9) U/lung (95% CI 177.6 to 381.4), bleomycin group 1000 (16.4) U/lung (95% CI 968 to 1032)). This increase was significantly reduced by losartan (3 days: bleomycin + losartan group 247.1 (28.6) U/lung (95% CI 198.5 to 295.7), 15 days: bleomycin + losartan group 869.4 (24.9) U/lung (95% CI 832.1 to 906.7)).

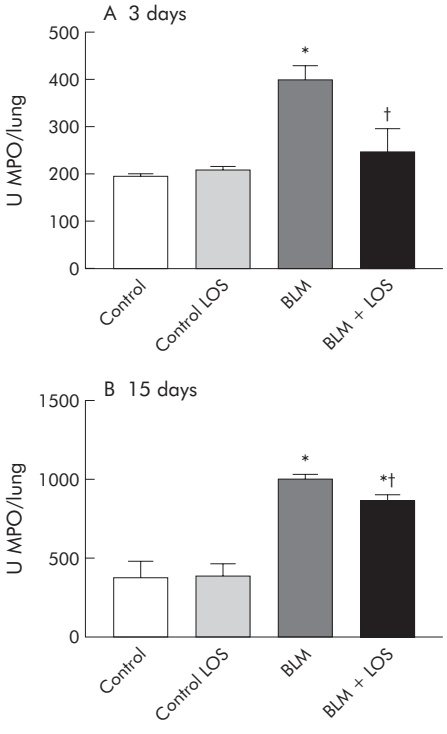

Figure 2 Effect of losartan (LOS) on lung inflammation induced by bleomycin (BLM) assessed by myeloperoxidase (MPO) activity (U/lung) in lung tissue at (A) 3 days and (B) 15 days. Bleomycin induced a rise in MPO activity at both 3 and 15 days, and this increase was inhibited by losartan (50 mg/kg). Statistical analysis was performed by one way ANOVA followed by Student's *t* test. *p<0.001 versus control group; †p=0.004 versus BLM group at 3 days; †p=0.005 versus BLM group at 15 days.

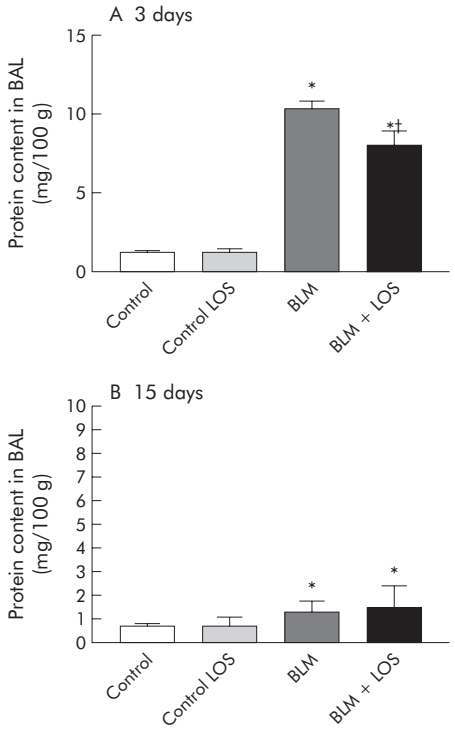

Figure 3 Effect of losartan (LOS) on lung inflammation induced by bleomycin (BLM) measured by protein content in BAL fluid (mg/100 g) at (A) 3 days and (B) 15 days. Protein in BAL fluid increased after BLM instillation on days 3 and 15 (*p<0.001 versus control group at 3 days; *p=0.04 versus control group at 15 days). At 3 days losartan reduced the total protein production initially induced by BLM (†p=0.04 versus BLM group). No differences were found between the BLM group and BLM + LOS group at 15 days (p=0.11, fig 3B). Statistical analysis was performed by one way ANOVA followed by Student's *t* test.

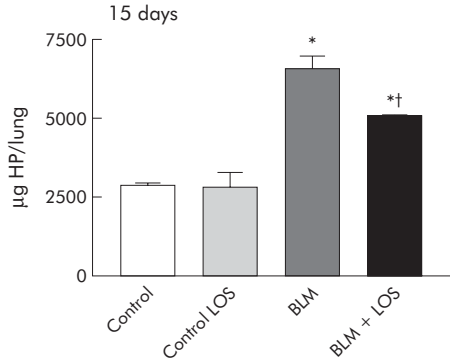

Figure 4 Antifibrotic effect of losartan (LOS) on bleomycin (BLM) induced lung fibrosis quantitatively measured using hydroxyproline (HP) content (μg/lung) at 15 days. The increased amount of HP content after BLM induction was inhibited by LOS. Statistical analysis was performed using one way ANOVA followed by Student's *t* test: *p=0.001 versus control group; †p<0.001 versus BLM group.

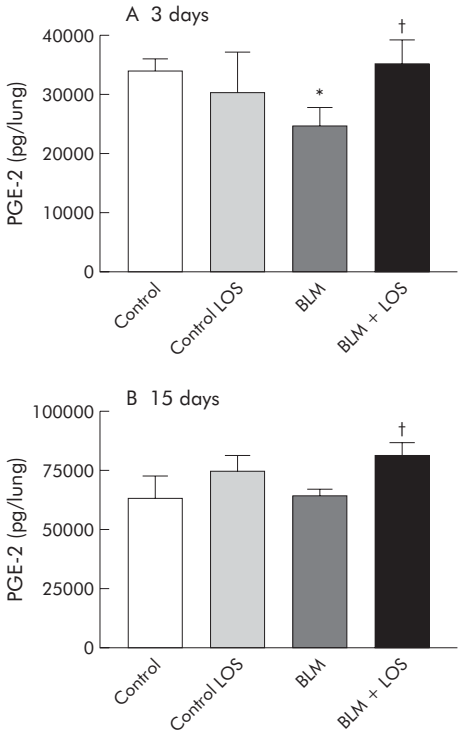

Figure 5 Effect of losartan (LOS) on synthesis of prostaglandin E_2 (PGE_2, pg/lung) at (A) 3 days and (B) 15 days after bleomycin (BLM) instillation. PGE_2 synthesis was reduced at 3 days and this reduction was prevented by losartan (*p = 0.01 versus control group; †p = 0.01 versus BLM group). At 15 days rats instilled with saline solution or BLM showed no differences in PGE_2 synthesis (p = 0.9), but LOS treatment upregulated PGE_2 production (†p = 0.004 versus BLM group). Statistical analysis was performed using one way ANOVA followed by Student's *t* test.

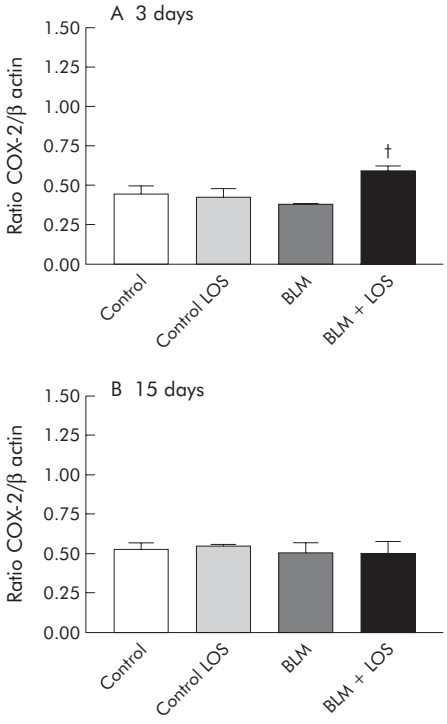

Figure 6 Effect of losartan (LOS) on lung COX-2 mRNA expression at (A) 3 days and (B) 15 days after bleomycin (BLM) instillation. The reduction in COX-2 expression after 3 days of BLM instillation was not significant. An increase in COX-2 expression was observed after 3 days of BLM instillation with LOS treatment (†p = 0.02 versus BLM group). At 15 days there were no differences between the BLM and BLM+LOS groups (p = 0.3). Statistical analysis was done by one way ANOVA followed by Student's *t* test.

Total recovery of BAL fluid typically exceeded 80%, and the percentages of fluid recovered did not differ significantly between experimental groups. Protein in BAL fluid was significantly increased after 3 days of bleomycin instillation, and this effect was inhibited by losartan treatment (control group: 1.2 (0.1) mg/100 g (95% CI 1 to 1.4); bleomycin group: 10.3 (0.5) mg/100 g (95% CI 9.4 to 11.2); bleomycin + losartan group: 8 (0.9) mg/100 g (95% CI 6.5 to 9.5), fig 3A). Fifteen days after bleomycin instillation the difference between the control group and bleomycin group was significant, but there were no differences between the bleomycin group and the losartan treatment group (control group: 0.7 (0.1) mg/100 g (95% CI 0.6 to 0.8); bleomycin group: 1.3 (0.1) mg/100 g (95% CI 1.1 to 1.5), fig 3B).

Lung HP content, a marker of collagen deposition, was increased at 15 days after exposure to bleomycin. This increase was significantly blocked by losartan, although levels remained higher than those found in animals not exposed to bleomycin (control group: 2875 (71) µg/lung (95% CI 2794.5 to 2955); bleomycin group: 6564 (414) µg/lung (95% CI 5838.4 to 7290); bleomycin + losartan group: 5081 (43) µg/lung (95% CI 5021.6 to 5140.6), fig 4).

Effect of losartan on PGE_2 and COX-2 mRNA levels

The concentration of PGE_2 in BAL fluid (pg/lung) was significantly inhibited by bleomycin instillation at 3 days. This effect was abrogated by losartan treatment which increased the level of PGE_2 (control group: 33849.1 (2065.6) pg/lung (95% CI 30985.9 to 36712.2); bleomycin group: 24673.7 (1516.7) pg/lung (95% CI 22015 to 27332.5); bleomycin + losartan group: 35076.3 (3986.6) pg/lung (95% CI 28309.6 to 41843.1), fig 5A). There were no significant differences in the amount of PGE_2 between control and BLM groups 15 days after fibrotic induction. Losartan treatment significantly increased the PGE_2 concentration at 15 days (control group: 63520.3 (9189.5) pg/lung (95% CI 47922.1 to 79118.4); bleomycin group: 64499.6 (2807) pg/lung (95% CI 59735 to 69264.1); bleomycin + losartan group: 81419.1 (5542.5) pg/lung (95% CI 71361.8 to 91476.4), fig 5B).

The expression of COX-2 mRNA was generally lower 3 days after bleomycin instillation. Fibrotic rats treated with losartan expressed a significantly increased amount of COX-2 mRNA at 3 days (control group: 0.44 (0.05) (95% CI 0.35 to 0.52); bleomycin group: 0.38 (0.003) (95% CI 0.37 to 0.38); bleomycin + losartan group: 0.59 (0.03) (95% CI 0.54 to 0.64), fig 6A). There were no changes in COX-2 mRNA expression at 15 days after bleomycin instillation and no significant differences were observed in the losartan group (control group: 0.53 (0.01) (95% CI 0.45 to 0.62); bleomycin group: 0.51(0.06) (95% CI 0.37 to 0.64); bleomycin + losartan group: 0.45 (0.08) (95% CI 0.28 to 0.62), fig 6B).

Histological features

Haematoxylin-eosin and Masson's trichrome stained lung sections were examined by light microscopy. Lungs from control groups at 3 and 15 days were histologically normal (data not shown). The administration of bleomycin resulted in characteristic histological changes including areas of inflammatory infiltration, thickening of alveolar walls (fig 7A) and an increase in interstitial collagen deposition and fibroblastic appearance (fig 7B). Losartan treatment significantly reduced the changes in lung morphology; there were fewer inflammatory infiltrates (fig 7C), less collagen deposition, and septal widening (fig 7D).

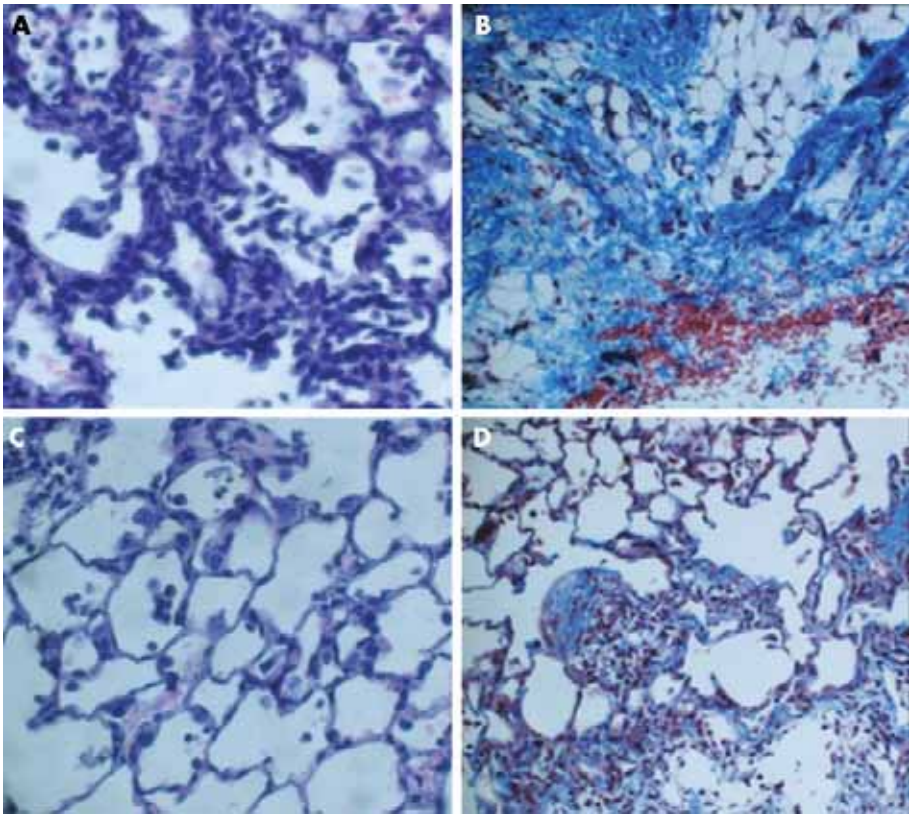

Figure 7 Histological examination of the anti-inflammatory and antifibrotic effects of losartan on bleomycin instilled lungs. Rats were instilled with bleomycin or saline solution. After 3 or 15 days of treatment with losartan they were sacrificed and the removed lungs stained with haematoxylin-eosin (H-E) or Masson-trichrome stain. (A) Fibrotic lung induced by bleomycin at 3 days and stained with H-E showing extensive interstitial inflammatory infiltration. (B) Fibrotic lung induced by bleomycin at 15 days and stained with Masson showing patchy areas of interstitial collagen deposition. (C) Fibrotic lung treated with losartan at 3 days and stained with H-E showing fewer infiltrates of inflammatory cells. (D) Fibrotic lung treated with losartan at 15 days and stained with Masson: although multifocal parenchymal lesions were also present, there was less septal widening and less collagen deposition. All fields were examined in each group. Original magnification: ×40.

DISCUSSION

We have shown that losartan, a selective AT1 receptor antagonist, ameliorates experimental lung fibrosis induced by bleomycin and increases PGE_2 synthesis. Our findings provide further evidence for the antifibrotic effect of losartan and support the potential role of PGE_2 as a protective molecule in lung fibrosis.

ANGII has been shown to be an important mediator in the pathogenesis of lung fibrosis and may influence the progression of lung injury via a number of mechanisms.[2 5] It has been reported that there is an increase in lung ANGII concentration, which precedes increases in lung collagen, and an upregulation in AT1 receptor expression in lung parenchyma after bleomycin induced lung injury.[11 12 25] The presence of a pulmonary renin-angiotensin system within the lung has been suggested several times in the literature. It has been reported that angiotensinogen and AT1 receptors are expressed in lung tissue.[26] Moreover, a high concentration of ACE in BAL fluid has been observed in patients with sarcoidosis and IPF.[27]

The study shows that losartan significantly decreased lung inflammatory infiltration both early (3 days) and late (15 days) after bleomycin exposure. The anti-inflammatory effect of AT1 receptor blockade has been reported in previous studies.[25 28 29] Our findings agree with those of Otsuka *et al*[25] who showed that candesartan cilexetil significantly decreases the concentration of neutrophils in BAL fluid in the bleomycin model of pulmonary fibrosis, suggesting that ANGII may play a role in neutrophilic infiltration in the lung. Our study also confirms that losartan is effective in reducing lung fibrosis induced by bleomycin. Controversially, a recent study by Keogh *et al*[30] has reported that losartan cannot attenuate the collagen deposition induced by bleomycin in mice. Differences in the animal model, the dose of bleomycin and losartan, and the time point of HP measurements may account for discrepancies between our results and those of Keogh *et al*.[30] Nevertheless, a large number of previous reports have shown that AT1 receptor antagonists decrease lung collagen deposition, in accordance with our observations.[11 25 31]

The mechanisms of the pulmonary antifibrotic effect of AT1 receptor antagonists have been investigated in recent years.[11–13 25] It is known that losartan inhibits the proliferation of human lung fibrotic fibroblasts induced by ANGII in vitro, through the activation of AT1 receptor and mediated by TGF-β.[12] Renzoni *et al* have reported that the ANGII receptor type 1 gene is one of the most relevant targets of TGF-β in primary lung fibroblasts from patients with IPF.[32] In the bleomycin model of pulmonary fibrosis, administration of losartan or candesartan cilexetil attenuates TGF-β expression and lung collagen deposition.[11 25] Similarly, other observations suggest that the antifibrotic effect of losartan could involve the regulation of TGF-β expression, providing further insights into the links between profibrotic mediators in the pathogenesis of pulmonary fibrosis. The present study was designed to investigate whether losartan can also act through antifibrotic mediators.

The most relevant finding in our study was the effect of losartan on PGE_2 synthesis and COX-2 expression. Losartan

significantly reversed the decrease in PGE_2 synthesis induced by bleomycin. A growing body of evidence supports the hypothesis that PGE_2 has a crucial role in the modulation of tissue repair and lung fibrosis.[33] PGE_2 inhibits fibroblast migration and proliferation in response to various mitogens and abrogates TGF-β induced collagen production.[16 34 35] In addition, fibrotic fibroblasts exhibit a marked reduction in the ability to upregulate PGE_2 synthesis in response to TGF-β, with the consequent loss of the antiproliferative response to TGF-β mediated by PGE_2.[17] Moreover, lung epithelial cells are a major source of PGE_2, and the capacity of these cells to inhibit fibroblast proliferation is related to their ability to produce PGE_2.[36] It appears that these changes are important in transforming the fibroblast from a relatively passive cell into a crucial component of IPF pathogenesis.[2] We hypothesised that the increased amount of ANGII may suppress the induction of PGE_2 synthesis, and this may result in an imbalance between profibrotic and antifibrotic mediators. Thus, reduction in the activity of ANGII would enhance the protective effect of PGE_2 in the fibrotic process. Our results confirm that AT1 antagonism increases PGE_2 and reduces collagen deposition. Taken together, these observations indicate that losartan acts on at least two of the most crucial mediators implicated in lung fibrosis, improving the balance between profibrotic (TGF-β_1) and antifibrotic (PGE_2) mediators.

The analysis of COX-2 mRNA expression showed that losartan reversed the reduction of COX-2 induced by bleomycin at 3 days, but no significant differences were found at 15 days. These results concur with studies in the kidney in which it was shown that ANGII inhibits COX-2 expression in rat renal cortex and that the administration of either captopril or losartan increases this expression.[37] However, the mechanisms involved in the regulation of COX-2 by ANGII in pulmonary fibrosis have not been investigated. The relevance of COX-2 as a protective mediator of pulmonary fibrosis has been demonstrated in vivo and in vitro.[17 38 39] COX-2 is the major source of the PGE_2 synthesised by alveolar epithelial cells.[36] The failure to synthesise PGE_2 in fibroblasts and lung tissue from patients with IPF has been shown to be associated with a decreased capacity to upregulate COX-2.[16–18] Interestingly, the increased PGE_2 level observed in lung fibrosis under losartan treatment did not correlate with a significant increase in COX-2 mRNA expression. This could be a consequence of the fact that AT1 antagonists also have an effect on other enzymes implicated in PGE_2 synthesis such as PGE_2 synthase, a hypothesis which requires future investigation.

In summary, our study shows that losartan enhances PGE_2 synthesis in experimental lung fibrosis and provides new insights into how AT1 receptor antagonists act against lung fibrosis. In addition, these data throw new light on the importance of ANGII as a regulator of the other main molecules implicated in the fibrotic process. This is a new approach to the molecular mechanisms implicated in the lung fibrotic process which could provide new insights for future treatments in lung fibrosis.

ACKNOWLEDGEMENTS

The authors thank Magda Mauri Cugat for her technical assistance and the animal facility carers at the University of Barcelona for their general support.

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This study was supported in part by grants from Hospital Clínic de Barcelona (Premi Fi de Residencia, 2003), SEPAR 2003, FIS 02/0186, FUCAP 2004, Red Respira (Fondo de Investigaciones Sanitarias, V-2003-REDC11D-0), and I+D SAF2003-04450.

Competing interests: none.

The study was approved by the institutional ethics committee and complied with European Community regulation (Directive 86/609/EEC) and Spanish guidelines for laboratory animal care.

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LUNG ALERT

Infants too young to receive pneumococcal conjugate vaccine benefit from herd immunity

▲ Poehling KA, Talbot TR, Griffin MR, *et al*. Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. *JAMA* 2006;**295**:1668–74

The Netherlands and the UK have recently joined a relatively small number of European countries to recommend a heptavalent pneumococcal conjugate vaccine (PCV7) as part of universal childhood immunisation programmes. PCV7 was incorporated into the US childhood vaccination programme in June 2000 and is recommended for all children aged 2–23 months. Since is introduction, evidence has shown that the rate of invasive pneumococcal disease (IPD) among children younger than 2 years has reduced by at least 60%. This study investigated for the first time rates of IPD in children aged 0–90 days before and after the introduction of PCV7.

A prospective population based study was undertaken with active laboratory based surveillance for IPD in infants aged 0–90 days across eight US states before (July 1997–June 2000) and after (July 2001–June 2004) the introduction of PCV7. The mean rates of IPD for infants aged 0–90 days decreased 40% from 11.8 (95% CI 9.6 to 14.5) to 7.2 (95% CI 5.6 to 9.4; $p = 0.004$) per 100 000 live births following PCV7 introduction. Notably, there was a significant decrease of 42% from 7.3 (95% CI 5.6 to 9.5) to 4.2 (95% CI 3.0 to 5.9; $p = 0.01$) per 100 000 live births in rates of IPD among infants aged 0–60 days—that is, those too young to receive the vaccine. Furthermore, subgroup analysis by race showed that the previous disproportionately high incidence of IPD in black compared with white infants was eliminated after the introduction of PCV7.

The authors conclude that this is the first study to suggest that neonates and infants too young to receive PCV7 are benefiting from herd immunity. They emphasise the importance of continued surveillance of IPD to observe if the trend continues, and to determine if serotypes not included in PCV7 emerge as an important cause of IPD in neonates and young infants.

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Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis

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Am J Physiol Lung Cell Mol Physiol 291:887-895, 2006. First published Jul 14, 2006;
doi:10.1152/ajplung.00432.2005

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Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis

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[1]*Department of Physiology, Michigan State University, East Lansing, Michigan; and* [2]*Servicio de Neumologia, Institut Clinic del Tórax, Hospital Clinic, Universidad de Barcelona;* [3]*Servicio de Anatomía Patológica, and* [4]*Departamento de Prologia Experimental, Institut de Investigaciones Biomèdiques, Consejo Superior de Investigaciones Cientificas, Barcelona, Spain*

Submitted 10 October 2005; accepted in final form 19 June 2006

Li, Xiaopeng, Maria Molina-Molina, Amal Abdul-Hafez, Jose Ramirez, Anna Serrano-Mollar, Antonio Xaubet, and Bruce D. Uhal. Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 291: L887–L895, 2006. First published July 14, 2006; doi:10.1152/ajplung.00432.2005.—Previous work from this laboratory demonstrated de novo synthesis of angiotensin (ANG) peptides by apoptotic pulmonary alveolar epithelial cells (AEC) and by lung myofibroblasts in vitro and in bleomycin-treated rats. To determine whether these same cell types also synthesize ANG peptides de novo within the fibrotic human lung in situ, we subjected paraffin sections of normal and fibrotic (idiopathic pulmonary fibrosis, IPF) human lung to immunohistochemistry (IHC) and in situ hybridization to detect ANG peptides and angiotensinogen (AGT) mRNA. These were analyzed both alone and in combination with cell-specific markers of AEC [monoclonal antibody (MAb) MNF-116] and myofibroblasts [α-smooth muscle actin (α-SMA) MAb] and an in situ DNA end labeling (ISEL) method to detect apoptosis. In normal human lung, IHC detected AGT protein in smooth muscle underlying normal bronchi and vessels, but not elsewhere. Real-time RT-PCR and Western blotting revealed that AGT mRNA and protein were 21-fold and 3.6-fold more abundant, respectively, in IPF lung biopsies relative to biopsies of normal human lung (both $P < 0.05$). In IPF lung, both AGT protein and mRNA were detected in AEC that double-labeled with MAb MNF-116 and with ISEL, suggesting AGT expression by apoptotic epithelia in situ. AGT protein and mRNA also colocalized to myofibroblast foci detected by α-SMA MAb, but AGT mRNA was not detected in smooth muscle. These data are consistent with earlier data from isolated human lung cells in vitro and bleomycin-induced rat lung fibrosis models, and they suggest that apoptotic AEC and myofibroblasts constitute key sources of locally derived ANG peptides in the IPF lung.

lung fibrosis; myofibroblast; alveolar epithelial cells; apoptosis

ANGIOTENSIN (ANG) II is known to play a key role in tissue fibrogenesis in a variety of organs including the heart, kidney, and liver (6, 26, 27). In experimental animal models of lung fibrosis, a key role for ANG II has been implicated by demonstrations that inhibitors of ANG converting enzyme (ACEI) or blockers of ANG receptor AT_1 could reduce or abrogate fibrogenesis in response to bleomycin (12, 22), monocrotaline (15), gamma irradiation (14), or the antiarrhythmic agent amiodarone (20). A key role for ANG II in the pathogenesis of lung fibrosis is further supported by the finding that knockout mice deficient in ANG receptor AT_{1a} are resistant to bleomycin-induced lung collagen deposition (8). The profibrotic potential of ANG II is believed to be mediated by upregulation of collagen gene expression in lung fibroblasts (12, 13), by induction of apoptosis in alveolar epithelial cells (AEC) (10), and other profibrotic actions (11).

Several lines of evidence suggest that the source(s) of precursor for the ANG II synthesis that drives fibrogenesis in the lung is generated locally, i.e., within the lung tissue itself. Cultured AEC of either human or rat origin synthesize angiotensinogen (AGT) mRNA and secrete ANG peptides on exposure to proapoptotic stimuli such as Fas ligand (24), TNF-α (21), or bleomycin (10). ANG II itself also induces apoptosis of AEC through ANG receptor AT_1 (25). In addition, myofibroblasts isolated from the lungs of patients with idiopathic pulmonary fibrosis (IPF) also synthesize AGT mRNA constitutively and secrete ANG peptides (23). The notion that local pulmonary synthesis of ANG II de novo, i.e., from the precursor AGT within the lung, is required for lung fibrogenesis is supported by the recent demonstration that intratracheal administration of antisense oligonucleotides against AGT mRNA could prevent bleomycin-induced lung fibrosis in rats without affecting circulating levels of AGT protein (9).

To date, the evidence in support of local pulmonary synthesis of AGT protein de novo is derived from either animal models (8, 9) or indirect studies of primary cells isolated from fibrotic human lung (23). In the present study it was therefore of interest to directly examine fibrotic human lung tissue in an attempt to identify local tissue sources of ANG peptide generation. We report here the findings of ANG peptide expression in at least two cell types, apoptotic AEC and myofibroblasts, in lung tissue from patients with IPF.

MATERIALS AND METHODS

Materials. Monoclonal antibodies to α-smooth muscle actin (α-SMA; clone 1A4), propidium iodide (PI), purified AGT from human serum, and biotinylated 2′-deoxyuridine 5′-triphosphate (Bio-dUTP) were obtained from Sigma (St. Louis, MO). Anti-cytokeratin monoclonal antibody (MAb) MNF-116 was purchased from Dako Cytomation (Carpinteria, CA). Antibodies recognizing AGT and ANG peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DNA polymerase I was obtained from Promega (Madison, WI). Biotinylated oligonucleotides for in situ hybridization (ISH) were obtained from Invitrogen (Carlsbad, CA). Solutions A and B of the Vectastain ABC Elite formulation were

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purchased from Vector Laboratories (Burlingame, CA). All other materials were of reagent grade.

Tissue samples and handling. Human lung tissue was obtained by open lung biopsy or video-assisted thoracoscopic surgery performed at Instituto del Tórax, Hospital Clínic de Barcelona. Fibrotic lung tissue was obtained from 12 patients with IPF; biopsies were obtained from more than one lung lobe. All patients had clinical, functional, and radiological features that fulfill the diagnostic criteria for an interstitial lung disease (ILD) (5). Briefly, all had progressive dyspnea, bilateral reticulonodular images on chest roentgenogram, restrictive lung functional impairment with decreased lung volumes and reduced single-breath carbon monoxide diffusing capacity, and hypoxemia at rest that worsened with exercise. Patients with IPF had neither antecedents of any occupational or environmental exposure nor any other known cause of ILD. None of the IPF patients had received steroids or other immunosupressant therapy at the time of clinical sample collection. Normal human lung tissue was obtained from individuals undergoing surgical treatment for spontaneous pneumothorax with no history of pulmonary disease. No histopathological evidence of disease was found in these tissue samples. Written informed consent was obtained from the patients according to institutional guidelines, and the study was approved by the Ethics Committee of Hospital Clínic de Barcelona.

All tissue was fixed in 10% neutral buffered formalin for 16 h and embedded in paraffin. Sections were cut at 5.0-μm thickness and mounted on glass coverslips. Human lung tissues designated for RNA isolation were immediately immersed in ice-cold TRI reagent (Molecular Research Center, Cincinnati, OH) after excision and were processed immediately. Lung tissues designated for analysis of proteins were flash-frozen in liquid nitrogen and stored at −80°C until protein isolation as described below.

In situ end labeling of fragmented DNA. Tissue sections were deparaffinized by passing through xylene, xylene-alcohol (1:1), 100% alcohol, and 70% alcohol for 10 min each. In situ end labeling (ISEL) of fragmented DNA was conducted with a modification of the method of Mundle et al. (16) performed as described previously in IPF lung tissue (18). Briefly, ethanol was removed by rinsing in distilled water for at least 10 min. The slides were then placed in 0.23% periodic acid (Sigma) for 30 min at 20°C. Samples were rinsed once in water and three times in 0.15 M phosphate-buffered saline (PBS) for 4 min each and were then incubated in saline sodium citrate solution (0.3 M NaCl and 30 mM sodium citrate in water, pH 7.0) at 80°C for 20 min. After four rinses in PBS and four rinses in *buffer A* [50 mM Tris·HCl, 5 mM MgCl, 10 mM β-mercaptoethanol, and 0.005% bovine serum albumin (BSA) in water, pH 7.5], the slides were incubated at 18°C for 2 h with ISEL solution (1.0 μM Bio-dUTP, 20 U/ml DNA polymerase I, and dATP, dCTP, and dGTP each at 0.01 mM in *buffer A*). Afterward the sections were rinsed thoroughly five times with *buffer A* and three additional times in 0.5 M PBS. For detections based on diaminobenzidine (DAB), the tissue was then incubated at 20°C with a solution consisting of 80 μl each of reagents A (avidin solution) and B (biotin-peroxidase solution) of the Vectastain Elite Kit (Burlingame, CA) in 3.84 ml of *buffer B* (1% BSA and 0.5% Tween 20 in 0.5 M PBS). After 30 min, the sections were washed four times in PBS and

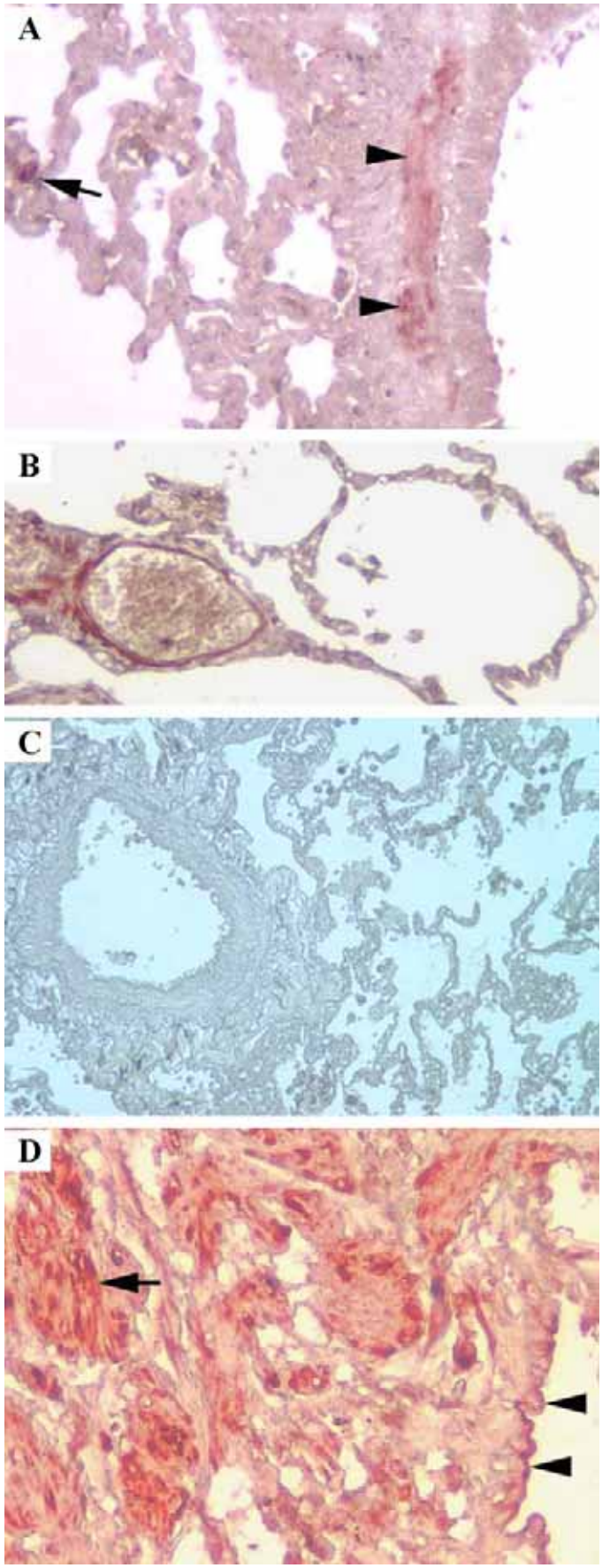


Fig. 1. Angiotensin (ANG) peptide immunoreactivity in normal and fibrotic human lung. Paraffin sections of histologically normal human lung tissue (*A* and *B*) and lung tissue from a patient with idiopathic pulmonary fibrosis (IPF) (*C* and *D*) were subjected to immunohistochemistry (IHC) with an antibody that recognizes ANG I and angiotensinogen (AGT). *A*: normal human lung, airway wall, and nearby parenchyma. Dark brown color = immunoreactivity. Arrowheads, smooth muscle underlying airways; arrows, occasional alveolar wall cells. *B*: normal human lung, vessel wall, and nearby parenchyma. *C*: normal human lung, same IHC procedure but with the primary antibody replaced by bovine serum albumin (BSA). *D*: human IPF lung, small air space, and nearby parenchyma. Arrow, possible myofibroblast focus; arrowheads, cuboidal epithelia. See subsequent figures for phenotype markers.

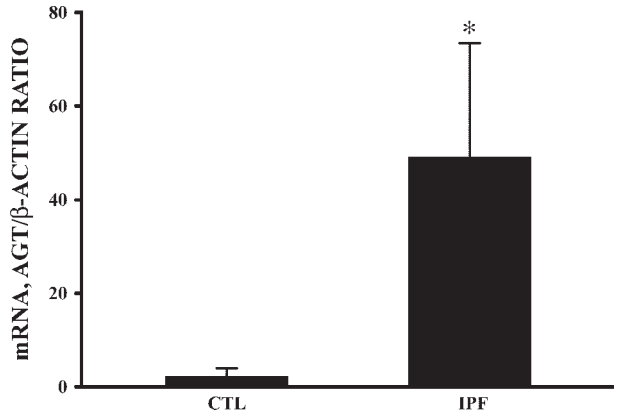


Fig. 2. Quantitative RT-PCR of AGT mRNA in normal and fibrotic human lung. Fresh IPF or normal control (Ctl) lung tissue was obtained by biopsy and immediately prepared for isolation of total RNA (see MATERIALS AND METHODS). Real-time RT-PCR was performed for both AGT and β-actin mRNAs as described in MATERIALS AND METHODS. Bars are means + SE of data collected from the biopsies of 4 (Ctl) and 5 (IPF) separate patients; $*P < 0.05$ by Mann-Whitney test.

were then immersed for 10 min in a solution of 0.25 mg/ml DAB in 0.05 M Tris·HCl, pH 7.5, containing 0.01% hydrogen peroxide. Alternatively, detection of incorporated dUTP was achieved with a Fast Blue chromogen system. The tissues were rinsed in distilled water three times and mounted under Fluoromount solution (Southern Biotechnology Associates, Birmingham, AL).

Immunohistochemistry and in situ hybridization. Immunohistochemistry (IHC) for ANG peptides, type II cell-specific cytokeratins, and α-SMA was performed with anti-ANG peptide antibody (Santa Cruz Biotechnology; 1:50 dilution), anti-cytokeratin antibody MNF-116 (Dako; 1:50 dilution), and an α-SMA-specific MAb (Sigma; 1:100 dilution). Deparaffinized lung sections were blocked with a solution of 3% BSA in PBS for 1 h; the primary antibody was then applied overnight at 4°C in 3% BSA-PBS. After a wash in PBS, the antibody was detected with a biotin-conjugated secondary antibody and avidin-linked chromogen system. Chromogens were either DAB (brown) or nitro blue tetrazolium (NBT; dark gray or black). For double labeling for ANG peptides and ISEL, ISEL was performed first as described above, and the next day ANG peptide primary antibody was applied for 2 h followed by detection with DAB chromogen. For double labeling with ANG peptide antibody and MNF-116 antibody, both primary antibodies were applied together overnight, and differential detection was achieved with anti-goat-horseradish peroxidase (HRP) secondary antibody (ANG peptide) or anti-mouse-AP (MNF-116) secondary antibody. Negative controls were obtained by completing the same procedure described above, but with omission of the primary antibody from the 3% BSA-PBS solution.

ISH was performed essentially as described previously (24). Deparaffinized slides were hybridized with digoxigenin-labeled antisense oligonucleotide DNA probes specific for AGT, which were detected with an amplified biotin-avidin system linked to NBT chromogen (purple). The digoxigenin-labeled probes used were 5′-AGGGT-GGGGGAGGTGCTGAACAGC-3′, as described by Lai et al. (7). A digoxigenin-labeled probe of the same base composition, but with scrambled sequence, was used as the control.

Microscopy and image acquisition. The prepared lung sections were photographed under transmitted or epifluorescent light on an Olympus BH2 epifluorescence microscope fitted with a SPOT Slider digital camera. Images of green fluorescence (α-SMA-FITC) were acquired through a 520-nm band-pass filter, and images of red fluorescence (PI) were acquired through a >570-nm long-pass filter.

RNA isolation and reverse transcriptase polymerase chain reaction. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously (21, 24), and real-time RT-PCR was performed in the Physiology Department of Michigan State University. The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. The identity of the PCR products was determined by expected size in 1.6% agarose gels and by DNA sequencing of the PCR product excised from agarose gels (not shown). Total RNA was extracted from biopsies with TRI reagent (Molecular Research Center) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{12–18}$. Real-time RT-PCR was performed with cDNA synthesized from 50 ng of total RNA, SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, and 0.2 μM specific primers for human AGT (forward 5′-GAG CAA TGA CCG CAT CAG-3′ and reverse 5′-CAC AGC AAA CAG GAA TGG-3′) and β-actin (forward 5′-AGG CCA ACC GCG AGA AGA TGA CC-3′ and reverse 5′-GAA GTC CAG GGC GAC GTA GC-3′), which produce PCR products of 151 and 332 bp, respectively. The PCR thermal profile started with 10-min activation of *Taq* polymerase at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 37 s, and extension at 72°C for 37 s, ending with dissociation curve analysis to validate the specificity of the PCR products. Reactions were performed in a Mx3000P machine (Stratagene, La Jolla, CA) and threshold cycle (C_T) data were collected with MxPro-Mx3000P software version 3.0. The relative AGT expression was normalized to β-actin and calculated with the comparative CT method of $2^{-\Delta\Delta CT}$.

Western blotting. Protein was extracted from biopsy samples by tissue homogenization in ice-cold Tris-buffered saline pH 8.0, supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and tributylphosphine. Soluble protein extracts (10 μg) were

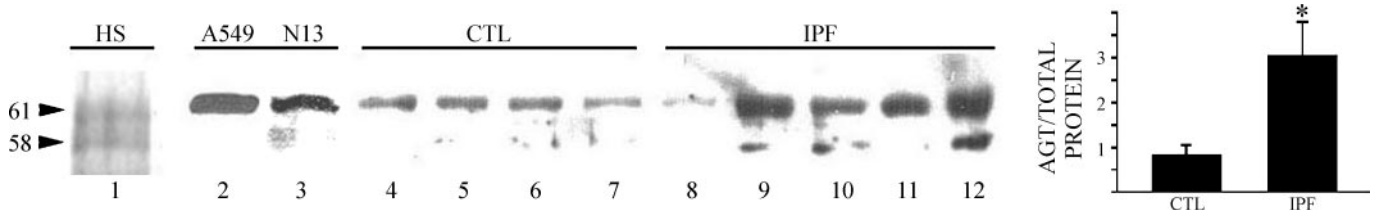


Fig. 3. Western blotting of AGT protein in normal and fibrotic human lung and isolated lung cells. Fresh IPF or normal control lung tissue was obtained by biopsy and immediately flash frozen for isolation of total protein (see MATERIALS AND METHODS). *Left*: Western blotting was performed as described in MATERIALS AND METHODS with the same antibodies used for IHC in Fig. 1; equal amounts of total protein per lane were loaded for *lanes 4–12*. *Lane 1*, positive control of AGT protein purified from human serum (HS); note 2 isoforms of AGT at ~58 and 61 kDa in similar abundance. *Lanes 2* and *3*, positive AGT protein controls of A549 cells (*lane 2*) or N13 primary human lung fibroblasts isolated from normal lung (*lane 3*; see Ref. 23). Note expression of high-molecular-mass isoform (top band, ~61 kDa) of AGT by isolated lung cells. *Lanes 4–12*, immunoreactive AGT in lung biopsies from normal (Ctl, *lanes 4–7*) or IPF (*lanes 8–12*) patients. *Right*: densitometry of 61-kDa AGT-to-total protein ratio, determined as described in MATERIALS AND METHODS. Bars are means + SE of data collected from biopsies of 4 (Ctl) and 5 (IPF) separate patients. $*P < 0.05$ by Student's *t*-test.

diluted 1:2 in Laemmli sample buffer (Bio-Rad, Hercules, CA), loaded on 10% Tris·HCl polyacrylamide gels, separated by SDS-PAGE, and then transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) in Towbin buffer. Blotting membrane was blocked by 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. Western blot analysis of AGT was performed with anti-ANG peptide antibody (1:400 dilution; Santa Cruz Biotechnology). Bands were visualized by HRP-conjugated donkey anti-goat secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology) and the chemiluminescent substrate Super Signal West Femto Maximum Sensitivity (Pierce, Rockford, IL). Images of the chemiluminescence-exposed film were analyzed for band intensity with Scion Image software (release beta 4.0.2) and normalized to total protein band intensities obtained by silver staining of SDS-polyacrylamide gels of replicate biopsy extracts. Silver staining was performed with a commercially available kit (Silver Stain Plus, Bio-Rad) according to the manufacturer's instructions.

Cell culture. The human lung cell line A549 was cultured as described previously (23–25). The primary human lung fibroblast strain N13, isolated from normal human lung, was recovered from cryostorage and cultured as described previously (23). Before analysis, cells were switched from growth medium containing fetal bovine serum to serum-free medium (Ham's F-12) for at least 2 days before harvesting. Immunoreactive AGT was detected by Western blotting of cells lysed in buffer containing NP-40 detergent and a commercially available protease inhibitor cocktail.

RESULTS

Earlier cell culture studies from this laboratory (21, 25) showed that apoptotic AEC or myofibroblasts isolated from IPF lung tissue synthesize ANG peptides de novo, i.e., from the precursor AGT. To begin determining whether these same cell types synthesize ANG peptides in the fibrotic human lung in situ, we subjected paraffin sections of histologically normal human lung tissue and lung tissue from a patient with IPF to IHC with antibodies that recognize ANG peptides; the specificity of the antibodies is discussed further in Fig. 3. As shown in Fig. 1, normal human lung (Fig. 1, *A–C*) showed immunoreactivity (dark brown color) in smooth muscle underlying airways (arrowheads) and occasional alveolar wall cells (arrow) but did not label in the absence of the primary antibody (Fig. 1*C*). Smooth muscle underlying large vessels of normal lung also was immunoreactive for ANG peptides (Fig. 1*B*, brown). In the human IPF lung (Fig. 1*D*), intense immunoreactivity was observed in numerous areas resembling fibroblastic foci throughout the parenchyma (arrows) and in cells resembling cuboidal epithelia within the surfaces of air spaces (arrowheads).

To obtain a quantitative assessment of ANG peptide expression in normal versus fibrotic human lung, we performed real-time RT-PCR for AGT and β-actin mRNAs on total RNA isolated from lung biopsies from five IPF patients and four patients without fibrotic lung disease. In Fig. 2, AGT mRNA was found to be 21-fold more abundant in IPF lung tissue relative to the control specimens of human lung ($P < 0.05$). Figure 3 shows quantitation of immunoreactive AGT in total protein extracts from a panel of lung biopsy specimens and purified human lung cells. By Western blotting with the same antibodies used for IHC, two bands of differing molecular mass, which represent the two isoforms of human AGT, were detected in a commercially available purified AGT standard isolated from human serum (Fig. 3, *lane 1*) and in IPF lung (Fig. 3, *lanes 8–12*). In human serum, the higher (~61 kDa)- and lower (~58 kDa)-molecular-mass isoforms of AGT were detected in apparently similar abundance. Cultured human lung epithelial cells (A549; Fig. 3, *lane 2*) and primary human lung fibroblasts (N13; Fig. 3, *lane 3*) expressed exclusively the 61-kDa isoform of AGT. Lung biopsies from both normal lung (Fig. 3, *lanes 4–7*) and IPF lung (Fig. 3, *lanes 8–12*) contained primarily the higher-molecular-mass isoform of AGT expressed by the isolated lung cells, although IPF biopsies did contain some of the low-molecular-mass isoform. Densitomet-

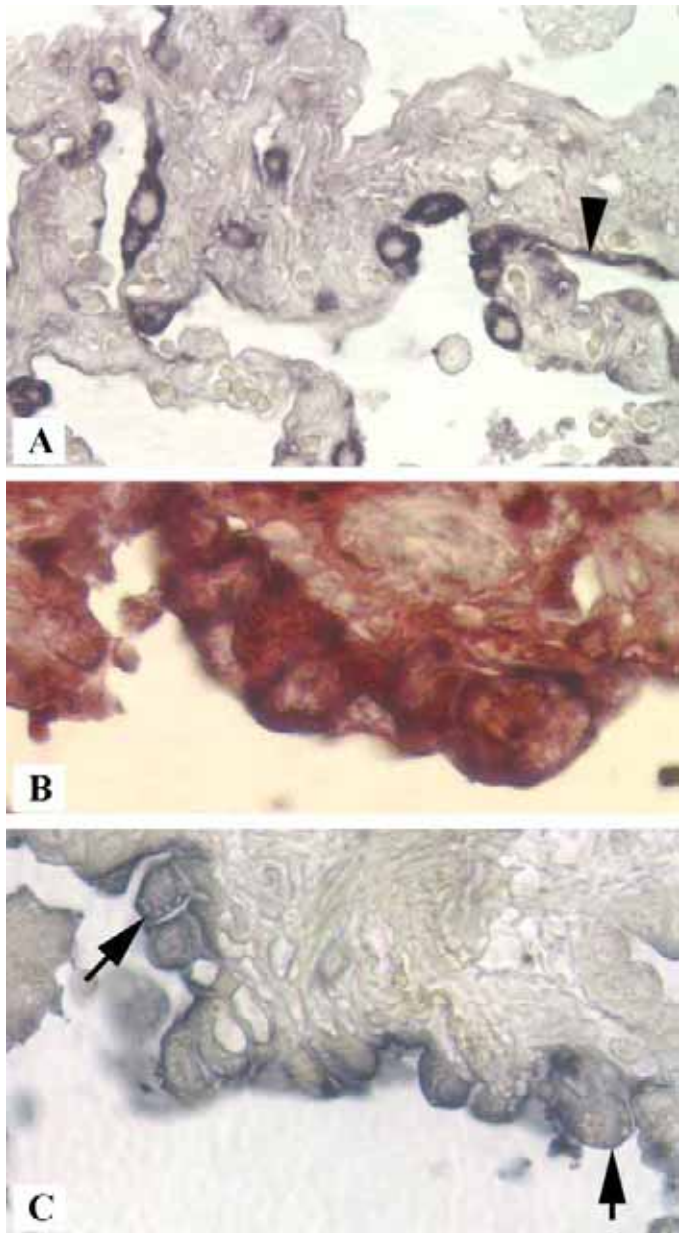


Fig. 4. Colocalization of ANG peptides with alveolar epithelial cell markers in IPF lung. Paraffin sections of lung tissue from a patient with IPF were subjected to IHC with an antibody that recognizes type II pneumocytes [monoclonal antibody (MAb) MNF-116, *A* and *B*] or with the ANG peptide antibody (*B* and *C*). *A*: immunoreactivity for MAb MNF-116 (black) in cuboidal epithelial cells and occasionally in attenuated cells in air space surfaces (arrowhead). *B*: double labeling with MAb MNF-116 and ANG peptide antibody showed ANG peptide immunoreactivity (brown) in MNF-116-positive cells (black). *C*: ANG peptide immunoreactivity in cuboidal epithelial cells containing perinuclear inclusion bodies (arrows).

ric quantitation of the higher-molecular-mass (~61 kDa) isoform of AGT only relative to total protein revealed 3.6-fold higher levels of the 61-kDa isoform of AGT in IPF lung biopsies compared to nonfibrotic lung ($P < 0.05$; Fig. 3).

Because of the severely altered structure of IPF lung, the determination of cell type in tissue sections is difficult if based on morphology alone. To begin identifying the cell types labeled by the ANG peptide antibodies in Fig. 1, we subjected lung tissue from a patient with IPF to IHC with an antibody that recognizes type II pneumocytes (MAb MNF-116; Ref. 4), applied alone or together with the ANG peptide antibody. In Fig. 4, immunoreactivity for MAb MNF-116 (Fig. 4*A*, black) was observed in cuboidal epithelial cells that were usually located in air space corners, or occasionally in attenuated cells in the air space surfaces (arrowhead). In Fig. 4*B*, double labeling with both MAb MNF-116 and ANG peptide MAb revealed ANG peptide immunoreactivity (brown) within MNF-116-positive cells (black). Figure 4*C* shows ANG peptide reactivity detected with an NBT-based chromogen system (dark gray) in cuboidal epithelial cells that appeared to contain lamellar bodies (arrows). In some regions, cells with the morphology of alveolar macrophages showed positive labeling with anti-ANG peptide antibody but negative reactivity with MNF-116 (not shown).

To determine whether ANG peptide expression by epithelial cells in IPF lung was associated with apoptosis in situ as it is in cell culture (21, 24), we subjected sections of normal human lung and sections from a patient with IPF to IHC with ANG peptide MAb, together with simultaneous double labeling by ISEL of fragmented DNA or PI plus DNase-free ribonuclease (PI-RNase). In Fig. 5*A*, double-labeled normal human lung revealed occasional ANG peptide-positive cells within alveolar walls (brown, arrowheads) or alveolar spaces (arrow), but very few ISEL-positive cells (blue). In contrast, double labeling of IPF lung (Fig. 5*B*) revealed numerous foci of cuboidal epithelia that were both ISEL positive (blue) and ANG peptide positive (brown) [note the ISEL-positive epithelial nuclei that also displayed chromatin condensation (arrowhead) or margination against the nuclear envelope (arrow), morphological hallmarks of apoptosis]. Fig. 5, *C* and *D*, show two views of the same microenvironment of IPF lung that was double-labeled with ANG peptide antibodies (*C*) and PI-RNase (*D*); paired black and white arrows highlight the condensed and marginated chromatin morphology of ANG peptide-positive cuboidal epithelial cells. For contrast, the normal nuclei of underlying stromal cells that are ANG peptide negative are denoted by

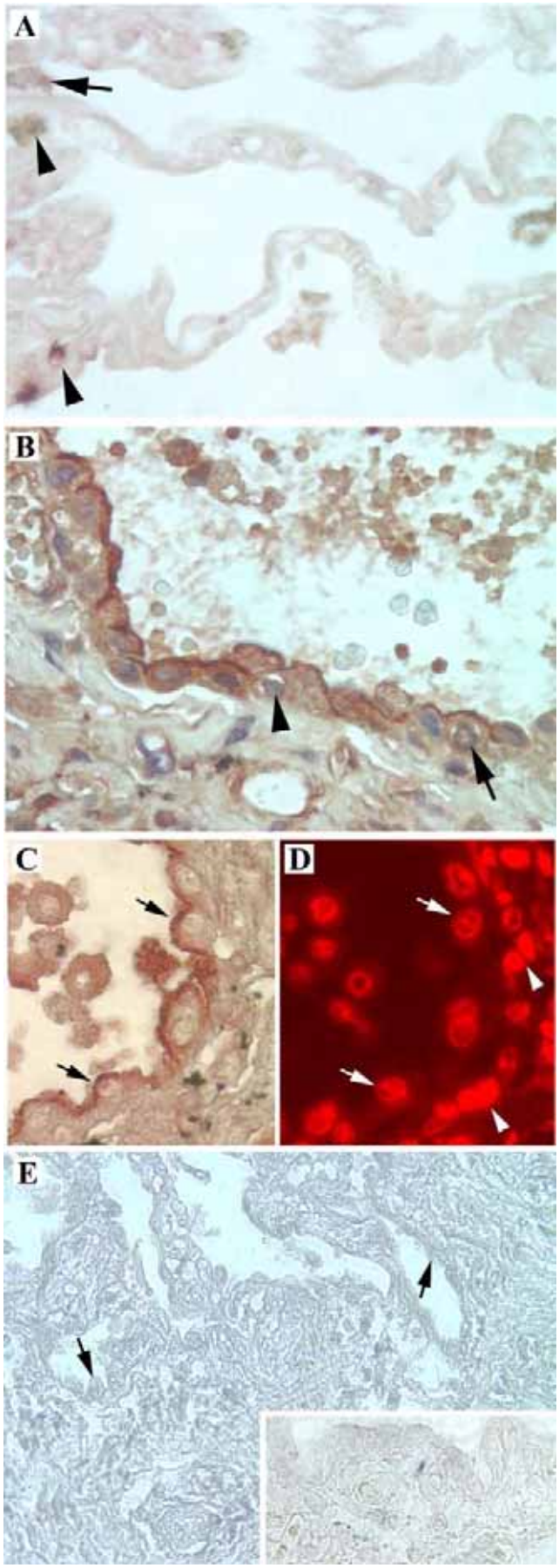


Fig. 5. Colocalization of ANG peptides with markers of apoptosis in epithelial cells within IPF lung. Lung sections from normal human lung (*A*) or from a patient with IPF (*B–E*) were subjected to IHC with ANG peptide antibodies with simultaneous double labeling by in situ end labeling of fragmented DNA (ISEL; *A* and *B*) or propidium iodide + DNase-free ribonuclease (PI-RNase) to highlight chromatin morphology (*C* and *D*). *A*: double-labeled normal human lung with occasional ANG/AGT-positive cells but very few ISEL-positive cells (blue). *B*: IPF lung showing foci of cuboidal epithelia that were both ISEL positive (blue) and ANG peptide positive; note chromatin condensation (arrowhead) or margination (arrow) in ISEL-positive nuclei. *C* and *D*: double labeling of IPF lung with ANG peptide antibody (*C*) and PI-RNase (*D*); note chromatin morphology of ANG peptide-positive epithelia (arrows, *C* and *D*) compared with normal nuclei in ANG peptide-negative stromal cells (arrowheads, *D*). *E*: negative labeling of IPF lung after IHC with the primary antibodies for ANG peptide replaced by BSA. Note lack of label in cuboidal epithelia (arrows). *Inset*: higher magnification of cuboidal epithelia.

arrowheads in Fig. 5*D*. Within the alveolar spaces, some of the cells labeled by ANG peptide antibody also had the morphology of alveolar macrophages (Fig. 5*C*) and did not colabel with the epithelial marker MNF-116 (not shown). Figure 5*E* shows no labeling of IPF lung on omission of the primary antibody.

Expression of ANG peptides de novo has been demonstrated in cultured myofibroblasts isolated from the IPF lung (23). To begin determining whether myofibroblasts within the IPF lung in situ also express ANG peptides, we subjected lung tissue from a patient with IPF to IHC with antibodies that recognize the myofibroblast marker α-SMA and ANG peptide. In Fig. 6, moderate immunoreactivity with α-SMA and ANG peptide antibodies (green and brown, respectively) was found in smooth muscle underlying vessels (paired white and black arrowheads, Fig. 6, *A* and *B*), but intense ANG peptide immunoreactivity was found in myofibroblast foci (paired arrows, Fig. 6, *A* and *B*). In Fig. 6, *C* and *D*, higher magnification revealed precise colocalization of ANG peptide immunoreactivity (*D*) with small microfoci of myofibroblasts (*C*), denoted by paired arrows. In Fig. 6*D*, the arrowhead denotes ANG peptide labeling within nearby epithelium.

ISH of IPF lung specimens (Fig. 7) for AGT mRNA revealed positive labeling in cuboidal epithelial cells and in occasional unidentified stromal cells (Fig. 7*A*). AGT mRNA also was detected in foci (Fig. 7*B*) that colabeled with antibodies against the myofibroblast marker α-SMA (Fig. 7*C*) applied to adjacent serial sections. In contrast, no evidence for AGT mRNA was detected in the smooth muscle underlying airways (Fig. 7*D*) or large vessels (Fig. 7*E*), nor was label deposited by scrambled-sequence control oligonucleotides (Fig. 7*F*). Little positive ISH signal was detected in normal lung (not shown).

DISCUSSION

Earlier in vitro studies from this laboratory (23, 24) showed that two cell types found in the fibrotic human lung, myofibroblasts and AEC undergoing apoptosis, were capable of synthesizing ANG peptides de novo, at least in cell culture. The studies presented here provide evidence from intact fibrotic human lung that these same cell types also synthesize ANG peptides in situ. The observations that airway and vascular smooth muscle are immunoreactive for ANG peptide antibodies (Fig. 1, *A* and *B*) agree with earlier findings by Ohkubo et al. (17) that ISH of rat lung detected AGT mRNA in fibroblast-like cells adjacent to vessels and bronchial walls. A subsequent investigation by Campbell and Habener (2) quantitated AGT mRNA in the lungs and showed that even though pulmonary expression of AGT is significantly less than that of liver, heart, or kidney, it is present in the lungs and is differentially regulated by nephrectomy or hormone treatment relative to other tissues.

Determinations of the mechanisms by which AGT expression is regulated in lung cells are the subject of ongoing investigations in this laboratory. Cultured AEC have been shown to synthesize ANG II in response to many proapoptotic stimuli in vitro, including Fas ligation (24), TNF-α (21), and bleomycin (10). For this reason we sought to determine whether ANG peptide expression might be found in AEC together with markers of apoptosis within the IPF lung. The localization of ANG peptide immunoreactivity in cells that bind anti-cytokeratin antibody MNF-116 and contain putative lamellar bodies (Fig. 4) supports the interpretation that at least some of the cells expressing ANG peptides are type II pneumocytes. Moreover, the finding of positive labeling by ISH for AGT mRNA in both cuboidal epithelia and α-actin-positive foci (Figs. 7, *A–C*) supports the contention that the ANG peptides are being synthesized de novo in AEC and myofibroblasts rather than being sequestered, for example, from other sources such as the blood. On the other hand, negative labeling for AGT mRNA in the smooth muscle underlying airway and vessel walls (Fig. 7, *D* and *E*) suggests that the positive IHC labeling of these cells (Fig. 1) may reflect uptake of ANG peptides rather than de novo synthesis.

Some cells with the morphology of alveolar macrophages and negative immunoreactivity to MNF-116 also were found to label with the ANG peptide antibody and by ISH for AGT

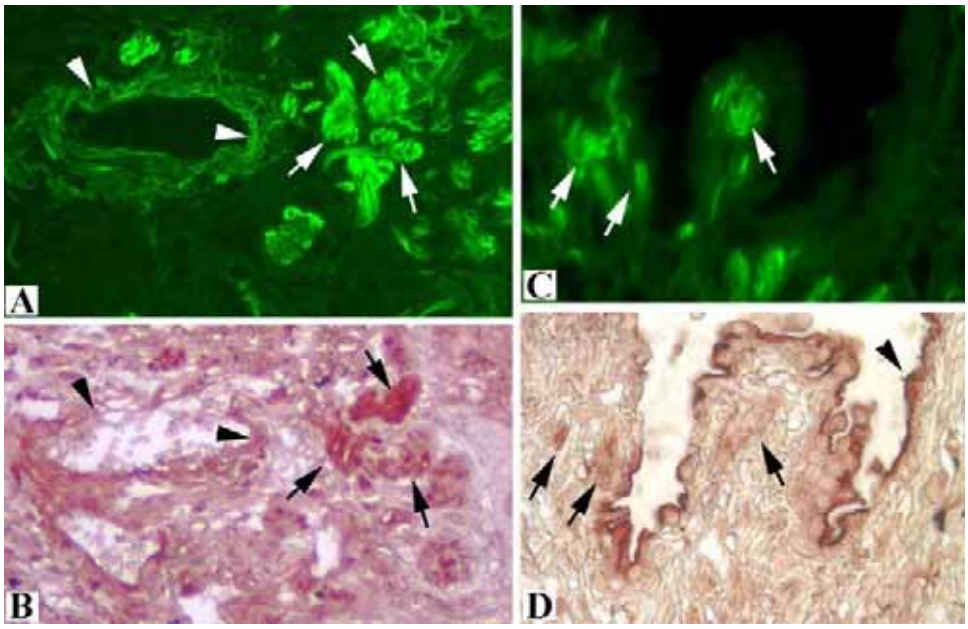
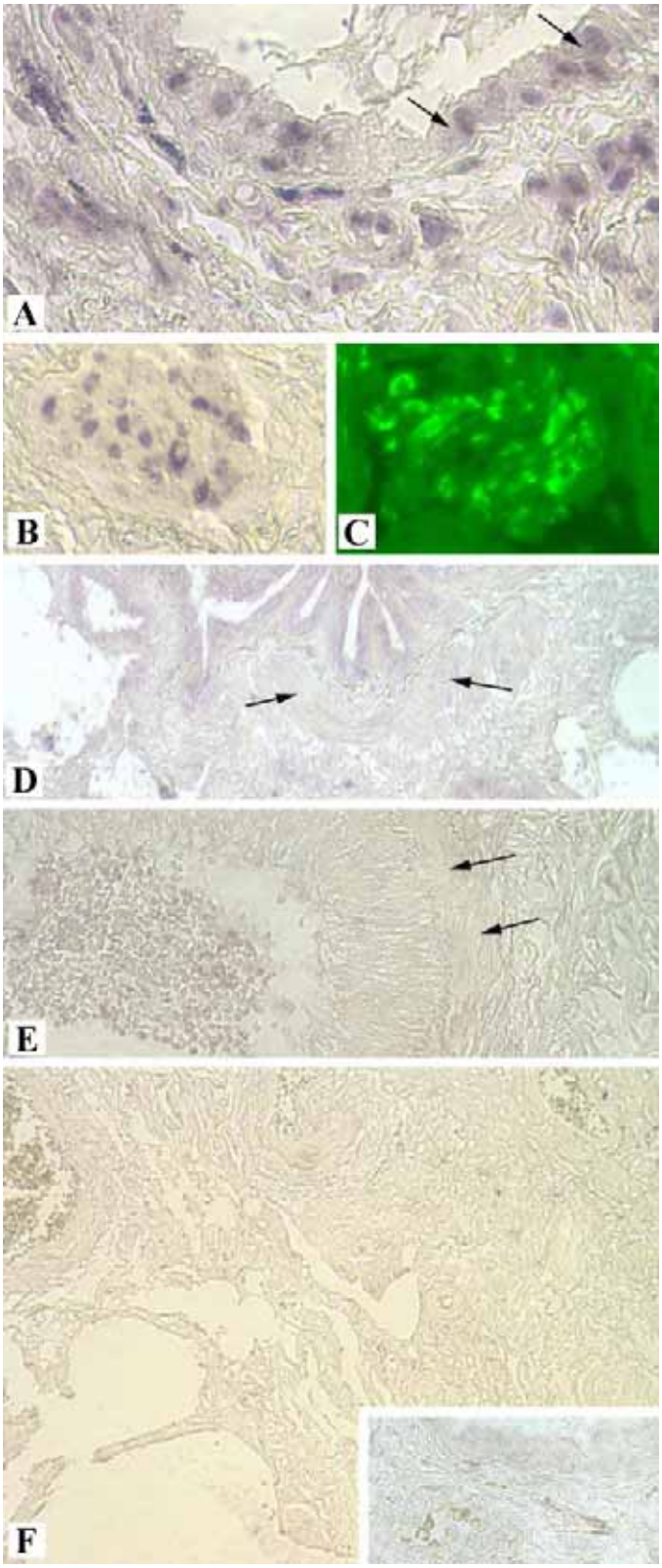


Fig. 6. Colocalization of ANG peptides with myofibroblasts in IPF lung. Lung tissue from a patient with IPF was subjected to IHC with an antibody that recognizes the myofibroblast marker α-smooth muscle actin (α-SMA MAb, green; *A* and *C*) or with ANG peptide antibody (brown; *B* and *D*). *A* and *B*: adjacent serial sections of IPF lung reveal mild ANG peptide MAb immunoreactivity in vessel smooth muscle (arrowheads) but intense ANG peptide immunoreactivity in myofibroblast foci (paired white and black arrows). *C* and *D*: higher magnification reveals precise colocalization of ANG peptide immunoreactivity (black arrows, *D*) with small microfoci of myofibroblasts (white arrows, *C*). In *D*, arrowhead denotes ANG peptide labeling in epithelial cells.

mRNA (not shown), but positive markers of macrophage phenotype were not available for positive phenotype analyses. Although far from definitive, these preliminary observations are consistent with the studies of normal human alveolar macrophages by Dezso et al. (3) and suggest that alveolar macrophages may also be a source of ANG peptides in fibrotic human lung. The possibility of ANG peptide expression by alveolar macrophages will be an interesting topic for further study.



The ANG peptide antibody used for these studies, which was derived against the peptide ANG I, recognizes ANG I, ANG II, and the two isoforms of AGT found in serum (Fig. 3). Given that the ~58- and ~61-kDa isoforms of AGT are both found in human serum, whereas isolated lung cells express only the 61-kDa isoform (Fig. 3), the finding of both isoforms in IPF lung biopsies suggests that some of the increase in lung tissue AGT in the IPF specimens may be due to serum-derived AGT. On the other hand, the observation that the 61-kDa isoform expressed by isolated lung cells was more highly abundant than the 58-kDa form in both normal and IPF lung supports the theory that most of the increase in lung tissue AGT in IPF occurs through de novo synthesis of AGT within the lung.

Because of the reactivity of the ANG peptide antibodies to AGT, ANG I, and ANG II, it is not possible to determine from the IHC studies alone whether the labeled regions contain primarily precursor AGT or the processed ANG peptides ANG I and ANG II. On the other hand, in vitro experiments with human lung myofibroblasts isolated from IPF biopsies (23) or cultured human or rat AEC (21, 24) show that both myofibroblasts and apoptotic lung epithelial cells can constitutively convert newly synthesized AGT to ANG II, apparently by autocrine mechanisms. Thus it seems likely that at least some of the immunoreactivity observed within foci of apoptotic AEC and myofibroblasts consisted of the processed peptides ANG I and ANG II. Determinations of the abundance of these processed peptides in human lung and the kinetics of their appearance will be interesting topics for future investigations.

Regardless, the detection of ANG peptide immunoreactivity in epithelial cells that simultaneously labeled positively for fragmented DNA by ISEL (Fig. 5*B*) and also exhibited chromatin condensation and margination against the nuclear envelope (Figs. 5, *C* and *D*) strongly suggests ANG peptide expression by apoptotic AEC in IPF lung in situ. Thus these findings are consistent with earlier studies of cultured AEC exposed to proapoptotic stimuli in vitro (21, 24). Nonetheless, the stimuli that caused the apoptosis of AEC detected in the IPF lung biopsies studied here are unknown. The observation that occasional epithelial cells in the normal human lung were found to

Fig. 7. In situ hybridization (ISH) for AGT mRNA in IPF lung. Lung tissue from a patient with IPF was subjected to ISH with antisense oligonucleotides that bind AGT mRNA (*A–E*) or with scrambled-sequence control oligonucleotides (*F*, see MATERIALS AND METHODS). *A*: in IPF lung, positive signal for AGT mRNA (purple) was observed in cuboidal epithelial cells lining many of the same air spaces that also labeled with ISEL (see Fig. 5). Labeling by ISH appeared to localize to both epithelial nuclei and cytosol (arrows, *A*) as well as in some unidentified stromal cells. *B*: positive signal for AGT mRNA (purple) in a putative focus of myofibroblasts. *C*: α-SMA immunolabeling of the same focus shown in *B*, but performed on an adjacent serial section. Note colocalization of ISH signal with α-SMA-positive cells. *D*: negative signal for AGT mRNA in smooth muscle (arrows) underlying an airway. *E*: negative signal for AGT mRNA in smooth muscle (arrows) underlying a large vessel. *F*: negative signal in IPF lung prepared for ISH with scrambled-sequence control oligonucleotides. *Inset*: higher magnification of cuboidal epithelia. Magnification = ×400 (*A*), ×200 (*B*, *C*, and *F inset*), ×100 (*D* and *E*), and ×50 (*F*); see RESULTS and MATERIALS AND METHODS for details.

be ANG peptide positive but ISEL negative (Fig. 5*A*) might be explained by the kinetics of apoptosis; the DNA fragmentation detected by ISEL is a relatively late downstream event in apoptosis (1), whereas AGT expression occurs within 2–3 h (9). Thus, depending on the timing of tissue fixation, not all ANG-positive cells would be expected to colabel by ISEL, and the images of fibrotic lung are consistent with that interpretation (Fig. 5*B*). Regardless, the findings herein also are consistent with an earlier investigation of IPF lung biopsies that revealed ISEL within epithelial cells adjacent to foci of myofibroblasts and heavy collagen deposition (7).

The findings that foci of α-SMA-positive cells also label heavily with the ANG peptide antibodies (Fig. 6) and AGT mRNA (Fig. 7, *B* and *C*) are consistent with other investigations showing ANG peptide expression by myofibroblasts of the fibrosing heart (26), kidney (6), and liver (27). They are also consistent with our earlier study (23) of myofibroblasts isolated from IPF lung biopsies, which synthesize and secrete ANG peptides in culture. Given that primary cultures of AEC undergo apoptosis on exposure to purified ANG II (25), TNF-α (21), or Fas ligand (24), it is difficult to know which of these proapoptotic factors were responsible for the induction of DNA fragmentation in AEC within the IPF biopsies studied here (Fig. 5).

In animal models, both ACEI (15, 22) and ANG receptor AT_1 antagonists (8, 14, 20) have been shown to prevent radiation- and chemical-induced experimental lung fibrosis; furthermore, knockout mice deficient in ANG receptor AT_{1a} are resistant to bleomycin-induced lung fibrosis (17). On that basis, it is speculated that the production of ANG peptides by apoptotic AEC and myofibroblasts is an important component of the molecular mechanisms that maintain a profibrotic environment within the IPF lung. Experiments are in progress to determine whether blockade of ANG receptors in short-term explant cultures of IPF lung tissue can reduce the expression of profibrotic genes.

In summary, immunolabeling and ISH studies of normal and fibrotic human lung have identified at least two extravascular sources, myofibroblasts and apoptotic AEC, that synthesize ANG peptides de novo in the IPF lung. These results confirm earlier investigations of cultured human AEC and myofibroblasts isolated from IPF tissue and are consistent with reports of local ANG-generating systems in other fibrosing organs. Given the roles of ANG II in stimulating collagen deposition in the lungs (8, 12) and other organs (6, 26, 27), it is theorized that the ANG peptides produced by apoptotic AEC and myofibroblasts contribute to the fibrogenic response in IPF lung. To evaluate this theory in human lung tissue, experiments designed to test for reduction of α-I-collagen mRNA in IPF lung explants by ANG receptor antagonists are currently under way.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-45136 and by grants from Instituto de Investigación Biomédica Agustí Pi Suñer, Fundación Respira-Sociedad Española de Neumología, and Fundación y Sociedad Catalana de Neumología ID, all of Spain.

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Angiotensin-TGF-β1 Crosstalk in Human Idiopathic Pulmonary Fibrosis: Autocrine Mechanisms in Myofibroblasts and Macrophages

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Abstract: Angiotensin II (ANGII) has been identified as a proapoptotic and profibrotic factor in experimental lung fibrosis models, and patients with the ID/DD polymorphism of ANG converting enzyme (ACE), which confers higher levels of ACE, are predisposed to lung fibrosis (Hum. Pathol. 32:521-528, 2001). Previous work from this laboratory has shown that human lung myofibroblasts isolated from patients with Idiopathic Pulmonary Fibrosis (IPF) synthesize the ANGII precursor angiotensinogen (AGT) constitutively. In attempts to understand the mechanisms and consequences of constitutive AGT synthesis by myofibroblasts, we studied myofibroblast-rich primary cultures of lung fibroblasts from patients with IPF (HIPF isolates), primary fibroblasts from normal human lung (NLFs), the IMR90 and WI38 human lung fibroblasts cell lines, and paraffin sections of lung biopsies from patients with IPF. Compared to the normal NLF isolates, HIPF primary fibroblast isolates constitutively synthesized more AGT and TGF-β1 mRNA, and released more AGT protein, ANGII and active TGF-β1 protein into serum-free conditioned media (both $p<0.01$). Incubation of HIPF fibrotic isolates with the ANGII receptor antagonist saralasin reduced both TGF-β1 mRNA and active protein, suggesting that the consitutive expression of AGT drives the higher expression of TGF-β1 by the HIPF cells. Consistent with this premise, treatment of either the primary NLFs or the WI38 cell line with 10^{-7}M ANGII increased both TGF-β1 mRNA and soluble active TGF-β1 protein. Moreover, induction of the myofibroblast transition in the IMR90 cell line with 2ng/ml TGF-β1 increased steady state AGT mRNA levels by realtime PCR (8-fold, $p<0.01$) and induced expression of an AGT promoter-luciferase reporter construct by over 10-fold ($p<0.001$). Antisense oligonucleotides against TGF-β1 mRNA or TGF-β neutralizing antibodies, when applied to the fibrotic HIPF cells in serum-free medium, significantly reduced AGT expression. In lung sections from IPF patient biopsies, immunoreactive AGT/ANGI proteins were detected in myofibroblasts, epithelial cells and presumptive alveolar macrophages. Together, these data support the existence of an angiotensin/TGF-β1 "autocrine loop" in human lung myofibroblasts and also suggest ANG peptide expression by epithelia and macrophages in the IPF lung. These findings may explain the ability of ACE inhibitors and ANG receptor antagonists to block experimental lung fibrosis in animals, and support the need for evaluation of these agents for potential treatment of human IPF. This manuscript discusses the data described above and their implications regarding IPF pathogenesis.

INTRODUCTION

The renin-angiotensin system (RAS) is now known to consist of a) the classically studied "Endocrine RAS", which largely regulates systemic blood pressure, and b) more recently characterized "Local RAS" systems that are observed in a wide variety of tissues. Local RAS systems, which have been identified in the heart, kidney, liver, lungs, brain, pancreas and adipose tissue [1-3], have been shown to function either in partial dependence on the endocrine RAS for some of its components (Local "Extrinsic" RAS systems), or may function independently of the endocrine RAS (Local "Intrinsic" RAS systems) through the synthesis of all RAS components in the local microenvironment [4].

In studies of tissue fibrogenesis, tissue RAS systems are believed to play a key role in both the initiation and progression of fibrous tissue accumulation in a variety of organs. For example, both angiotensin converting enzyme inhibitors (ACEi's) and angiotensin (ANG) receptor antagonists (ARAs) have been shown to prevent fibrogenesis of the heart [5], liver [6], kidney [7] and lungs [8,21,29] by mechanisms shown to be independent of hemodynamic parameters through the use, for example, of tissue explants in long term culture [9,10]. Investigations involving genetic manipulation have agreed with these findings; overexpression of the gene for angiotensinogen (AGT) in mice causes renal fibrosis [2], whereas deletion of the AGT gene attenuates experimental obstructive nephropathy [1]. Similarly, deletion of the ANG receptor AT1a gene in mice attenuates bleomycin-induced lung injury and fibrosis [9]. In each of these settings, the myofibroblast (or in liver, the hepatic stellate cell) is believed to be a key player in matrix deposition due to its colocalization with regions of extracellular matrix (ECM) deposition and the increased rates of ECM component synthesis displayed by these cells [11].

A link between myofibroblasts and the local RAS was established by demonstrations that these cells, in contrast to normal fibroblasts, synthesize AGT and other RAS components. Constitutive expression of AGT by myofibroblasts has been demonstrated *in vitro* after isolation of these cells from the fibrosing rat heart [12-14], from the cirrhotic human liver [15], or from the lungs of patients with Idiopathic Pulmonary

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Fibrosis [16]. Myofibroblasts also synthesize AGT and ANGII *in vivo* in the fibrosing mouse kidney [17]. A profibrotic role for the AGT and ANGII produced by these cells has been shown by clear demonstrations that ANGII upregulates both TGF-β1 and type I collagen gene expression in primary fibroblasts isolated from the kidneys [18], heart [19], liver [6] and human lungs [20,21]. In the light of these observations from a variety of organ fibrosis models and the known critical role of TGF-β1 in lung fibrosis models [22], it was of interest to investigate further the interrelationships between angiotensin, TGF-β1 and myofibroblasts in Idiopathic Pulmonary Fibrosis (IPF).

ANGIOTENSIN GENERATION BY HUMAN LUNG MYOFIBROBLASTS

Earlier studies of the soluble factors secreted by lung fibroblasts lead to the finding that fibroblasts isolated from the lungs of patients with IPF secrete a factor that induces apoptosis in cultured alveolar epithelial cells [16]. Further analyses identified the factor as angiotensin II, which by itself induces apoptosis when applied to primary cultures of lung alveolar epithelial cells [23]. The IPF cell isolates, which contained a high fraction of myofibroblasts identified by immunolabeling of alpha-smooth muscle actin (α-SMA), were found to synthesize AGT mRNA and to secrete immunoreactive AGT protein and the mature peptide ANGII [16]. The ANG peptides were detected in much higher amounts than those made by fibroblast isolates from normal human lung, which also contained a small number of α-SMA-positive cells and produced a basal level of ANG peptides [16].

In the interest of studying the IPF cells further, primary isolates of normal human lung fibroblasts (NLF) and IPF lung cell isolates (HIPF) were cultured for additional analyses of the RAS and TGF-β1. Fig. (**1**) shows α-SMA immunolabeling of the NLF fibroblast and HIPF myofibroblast primary cultures studied below; note that although the HIPF cultures are predominantly α-SMA-positive, the NLF cultures also contain occasional α-SMA-positive cells. As reported earlier [16], the HIPF cultures constitutively synthesize AGT mRNA at much higher levels than NLF cultures (Fig. **2A**), even under the unstimulated conditions of serum-free medium. Moreover, the HIPF cells expressed a transiently transfected AGT-promoter luciferase reporter construct (Fig. **2B**), which also was transcribed highly (relative to the NLFs) even in serum-free medium.

PROFIBROTIC ACTIONS OF LOCAL ANGIOTENSIN SYNTHESIS IN THE LUNG

To confirm and extend the findings of Marshall *et al*. [21] that ANGII can unpregulate TGF-β1 and collagen synthesis in normal human lung fibroblasts, ANGII was applied in serum-free medium to the primary NLF cultures or to the normal human lung fibroblast cell lines WI-38 and IMR-90. In Fig. (**3**), ANGII increased the amount of active TGF-β1 protein or TGF-β1 bioactivity released into serum-free media by the primary NLF cells (Panel A) or by the WI-38 cell line (Panel B), respectively. In both cases, the increase was blocked by the ANG receptor AT1-selective antagonists L158809 or losartan (LOS). In Panel C, ANGII increased TGF-β1 mRNA transiently at 6 hours after application to the IMR-90 cell line. Together, these data are consistent with the demonstration by Marshall *et al*. [21] that ANGII increases collagen gene expression in normal human lung fibroblasts through AT1 receptor-mediated upregulation of TGF-β1 synthesis in the fibroblast itself.

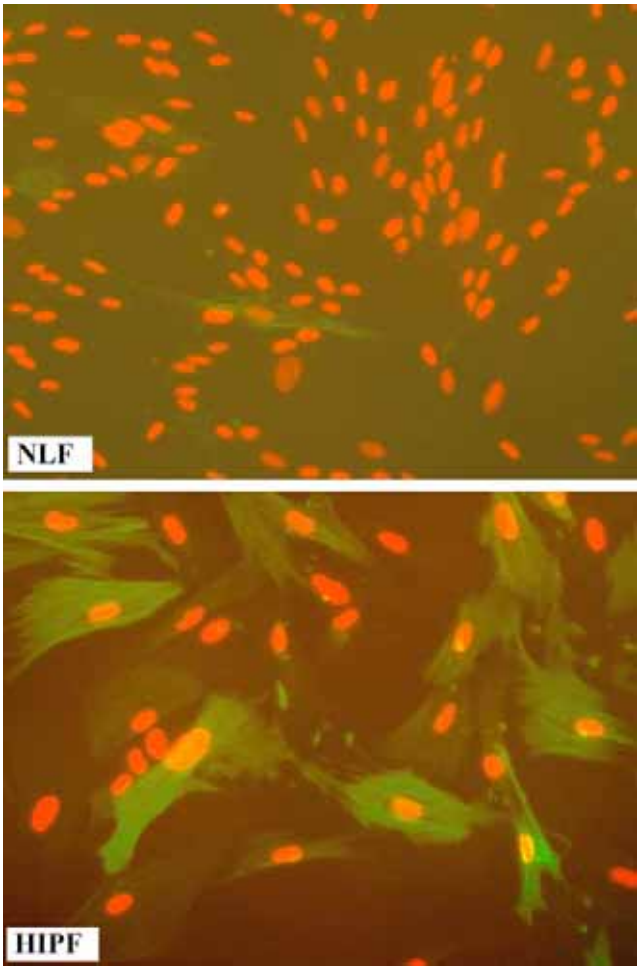

Fig. (1). Immunofluorescence visualization of myofibroblasts in primary cultures of normal and fibrotic human lung fibroblasts. Fibroblasts were isolated from histologically normal human lung (NLF, top panel) or from biopsies obtained from patients with Idiopathic Pulmonary Fibrosis (HIPF, lower panel) as described earlier [16]. Ethanol-fixed cells were immunolabeled with FITC-conjugated antibodies against alpha-smooth muscle actin (α-SMA), a myofibroblast marker [26]. Note prominence of α-SMA-positive cells in HIPF primary cultures, but only occasional appearance in NLF cultures. Red is propidium iodide counterstain of cell nuclei. Magnification 200X (both panels).

In earlier studies of a panel of NLF and HIPF primary cell isolates [24], Ramos *et al*. showed that HIPF myofibroblast isolates constitutively express more TGF-β1 and type I collagen mRNA, relative to normal NLF fibroblast isolates. Fig. (**4A**) extends those results by showing that the HIPF cells release more active TGF-β1 protein into serum-free media, on a per cell basis, than do the NLF primary isolates. These findings, combined with the above demonstration that

HIPF cells also constitutively express AGT and ANGII, lead to the hypothesis that the constitutive expression of ANG peptides might *drive* the increased basal expression of TGF-β1 and type I collagen in the HIPF myofibroblasts.

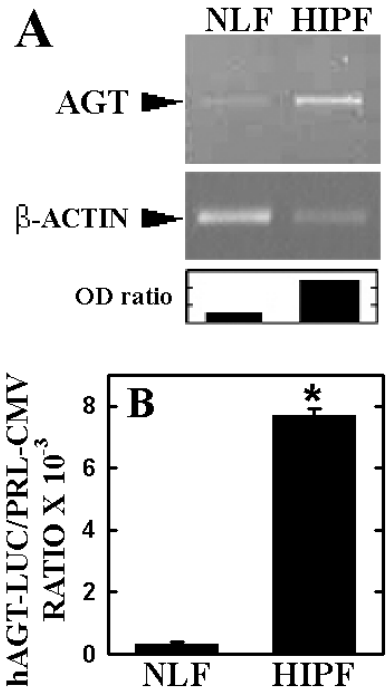

Fig. (2). Constitutive expression of angiotensinogen (AGT) mRNA and an AGT promoter-luciferase reporter by human lung myofibroblasts. A: Primary fibroblasts from normal (NLF) and fibrotic (HIPF) human lung were cultured in serum-free medium and were subjected to conventional RTPCR for AGT and β-actin as described earlier [32]. Bars are the AGT/β-actin ratio calculated by densitometry. B: Primary human lung fibroblast cultures were cotransfected with a full-length human AGT promoter-luciferase reporter (hAGT-LUC, detected as firefly luciferase) and a control plasmid (detected as renilla with the Dual Luciferase Reporter Assay System, Promega Corp., Madison WI). AGT-luciferase activity under serum-free conditions is expressed relative to that of the control plasmid driven by the CMV promoter (PRL-CMV). Bars are the mean ± S.E.M. of at least three fibroblast cultures; * = $p<0.01$ versus NLF by Student Newman Keul's test.

To test this theory, HIPF cells were challenged with the nonselective ANG receptor antagonist saralasin (SARAL) over 72 hours in serum-free media to determine if blockade of autocrine ANGII function might reduce TGF-β1 and collagen expression. As anticipated, the ANG receptor blocker reduced the release of TGF-β1 active protein (Fig. **4A**) and decreased the steady-state levels of mRNA for both TGF-β1 and type I collagen (Fig. **4B**). Thus, the constitutive expression of AGT and ANGII by human lung myofibroblasts appears to play a key role in maintaining increased autocrine expression of TGF-β1 and collagen by the cells. This mechanism *in vivo* might be important in maintaining a profibrotic poise within local microenvironments devoid of other profibrotic mediators released, for example, by inflammatory cells; indeed, inflammation often is not a prominent feature in IPF lung tissue [25].

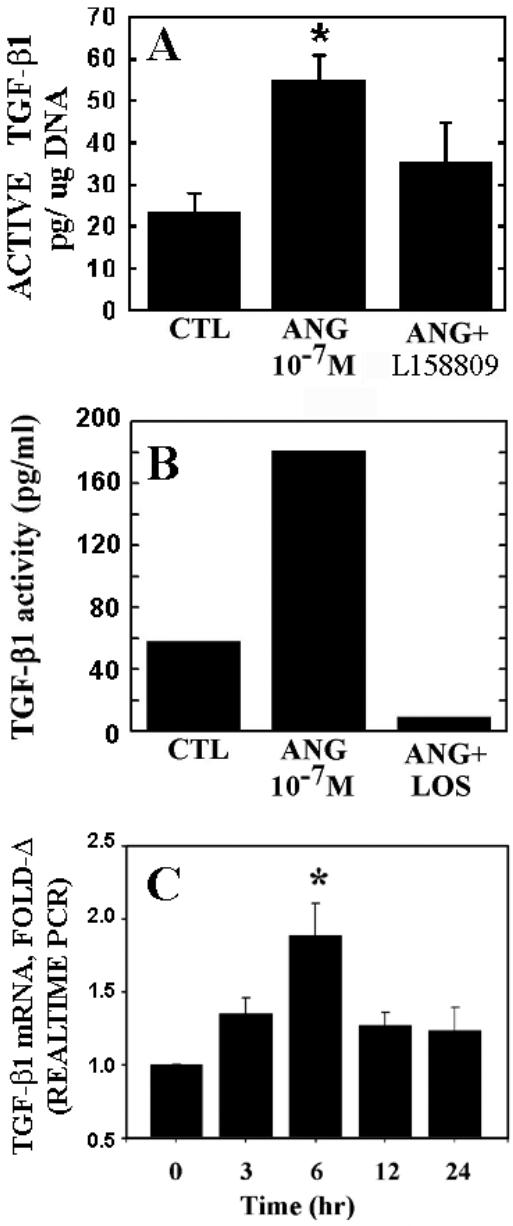

Fig. (3). Angiotensin II upregulates TGF-β1 mRNA and active protein in normal human lung fibroblasts. Angiotensin II (ANG) was applied in serum-free medium to primary cultures of NLFs (A) or to the normal human lung fibroblast cell lines WI-38 (B) or IMR-90 (C), in the presence or absence of the ANG receptor AT1 antagonists L158809 or losartan (LOS) at 10^{-6}M. The data displayed are (A) TGF-β1 active protein by ELISA, (B) TGF-β1 activity by Mv1Lu bioassay [33], or (C) TGF-β1 mRNA by realtime PCR. Note inhibition of ANG-induced TGF-β activation by the receptor AT1 antagonists. Bars are the mean ± S.E.M.; * = $p<0.05$ versus control (CTL) or time 0 by Student Newman Keul's test.

A PROFIBROTIC ANGIOTENSIN-TGF-β1 AUTOCRINE LOOP IN HUMAN LUNG MYOFIBROBLASTS

In light of the commonality of AGT expression by myofibroblasts from a variety of tissues [12-17] and the known profibrotic roles of ANGII [18-21], the definition of the factor(s) which maintain increased basal AGT expression by

myofibroblasts is a high priority. TGF-β1 is considered one of the most potent stimuli for normal fibroblasts to undergo the transition to myofibroblasts [26]. On this basis, it was

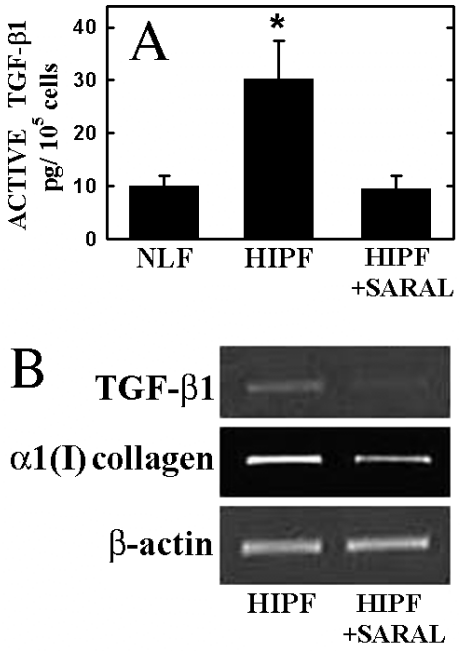

Fig. (4). Constitutive expression of active TGF-β1 by human lung myofibroblasts is downregulated by ANG receptor blockade. Normal (NLF) and fibrotic (HIPF) human lung fibroblasts described in Figs 1 and 2 were cultured in serum-free medium in the presence and absence of the nonselective angiotensin receptor blocker saralasin (SARAL, 50ug/ml). A: Active TGF-β1 protein was measured by specific ELISA in serum-free media conditioned over 72 hours. B: Semiquantitative RTPCR was performed on total RNA isolated from the cells described in Panel A. Note reduced TGF-β1 mRNA and active protein, and reduced α1(I)collagen mRNA in HIPF cells treated with saralasin. Bars are the mean ± S.E.M. of n = 4; * = p<0.05 versus NLF by Student Newman Keul's test.

theorized that TGF-β1 might itself upregulate AGT gene expression during the transition of normal lung fibroblasts to the myofibroblast phenotype. To test this theory, the normal human lung fibroblast cell line IMR90 was challenged with 2ng/ml TGF-β1 in serum-free media. Fig. (**5A**) shows that the myofibroblast marker α-SMA increased steadily with TGF-β1 treatment and confirmed the induction of the myofibroblast transition in IMR90 cells. Moreover, the steady state level of mRNA for AGT also increased with TGF-β1 treatment and reached a maximum increase of eight-fold by 48 hours (Fig. **5B**), as assessed by realtime PCR. Fig. (**5C**) shows that TGF-β1 also increase the expression of an AGT-promoter luciferase reporter construct, which suggests that the upregulation occurred at the level of AGT transcription. These data demonstrate that TGF-β1 induces AGT expresiosn in normal human lung fibroblasts.

This finding raised the possibility that increased basal expression of TGF-β1 by the HIPF myofibroblasts (see Fig. **4A** and ref. [24]), which is driven by autocrine ANGII function (Fig. **4**), might in turn *drive* the increased autocrine expression of AGT. To examine this possibility, primary HIPF myofibroblasts were challenged with antagonists of TGF-β1 function to determine if they might decrease the steady-state level of mRNA for AGT. In Fig. (**6**), AGT mRNA in the primary HIPF cells was reduced by neutralizing antibodies against TGF-β1 protein (anti-TGF-β) and by antisense oligonucleotides against TGF-β1 mRNA (AS), but was not reduced by scrambled-sequence control oligonucleotides (SCR) or by isotype-matched nonimmune control antibodies (NS IgG). These data and those of Fig. (**5**) suggest that autocrine expression of TGF-β1 by HIPF myofibroblasts drives their autocrine expression of AGT, and vice versa.

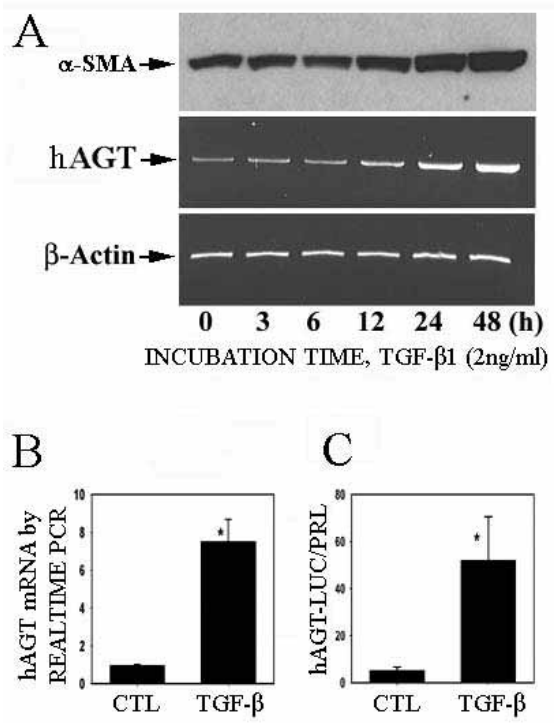

Fig. (5). TGF-β1 upregulates AGT mRNA and an AGT promoter-luciferase reporter in normal human lung fibroblasts. The IMR90 normal human lung fibroblast cell line was incubated in serum-free medium supplemented with active TGF-β1 for up to 48 hours.

A: Western blotting and RTPCR of alpha-smooth muscle actin (α-SMA) protein and AGT mRNA with TGF-β1 treatment. B: Realtime PCR of AGT mRNA at 48 hours of TGF-β1 treatment. C: Activity of the human AGT promoter luciferase reporter at 48 hours of TGF-β1 treatment. Bars are the mean ± S.E.M. of n = 4; * = p<0.05 versus NLF by Student Newman Keul's test.

Together, the data described above have lead to the Working Hypothesis displayed in Fig. (**7**) (see Fig. **7** Legend for details). In this model, local upregulation of either angiotensin or TGF-β1 expression can induce the other, and antagonists of either player can halt, at least theoretically, the progression of the proposed "autocrine loop" of ANGII/ TGF-β1 synthesis. Data from whole animal models support the plausibility of this model by confirming that antagonists of either TGF-β1 [27,28] or ANGII [9,10,21,29] can block the appearance of myofibroblasts and prevent lung collagen accumulation. Testing of the theory with explants of fibrotic human lung tissue *in vitro* is now underway. A key issue in the successful application of similar strategies to the human

lung *in vivo* will be the testing of antagonist delivery approaches designed to maximize local drug availability, such as aerosols or the intratracheal antagonist instillation methods that were successful in blocking lung fibrogenesis in mice [9].

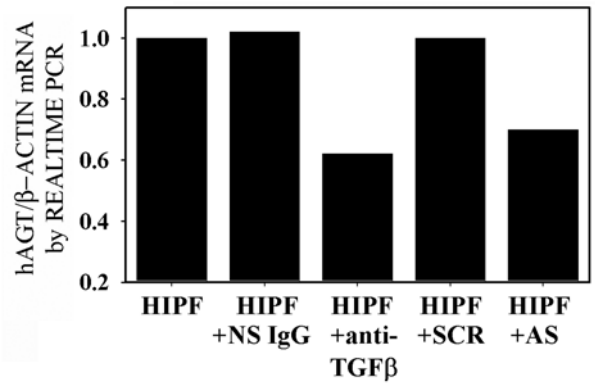

Fig. (6). Inhibition of constitutive AGT expression by blockade of autocrine TGF-β1 in human lung myofibroblasts. Primary fibroblasts from fibrotic (HIPF) human lung were cultured in serum-free medium with and without TGF-β1-neutralizing antibodies (anti-TGFβ), isotype- matched control antibodies (NS IgG), or with antisense (AS) or scrambled control (SCR) oligonucleotides against TGF-β1 mRNA [34]. After 24 hours, cells were harvested and subjected to realtime PCR for AGT and β-actin. Bars are the mean of two experiments. Note decrease in steady state AGT mRNA with TGF-β1 neutralizing antibodies or antisense oligonucleotides.

LOCALIZATION OF AGT EXPRESSION IN FIBROTIC HUMAN LUNG

In the interest of confirming that myofibroblasts in fibrotic human lung *in situ* express AGT as they do *in vitro*, paraffin sections of lung biopsies from patients with IPF were immunolabeled with antibodies that recognize the myofibroblast marker α-SMA, or with antibodies that recognize AGT protein and its product ANGI peptide [10]. Fig. (**8A**) shows at least three microfoci of α-SMA immunolabeling (white arrows) within an IPF lung biopsy, that were not associated with bronchial or vessel walls and thus likely indicate myofibroblasts. Fig. (**8B**) shows the same cellular microenvironments in a serial section of the same sample, but after immunolabeling with the AGT/ANGI antibodies (brown). The black arrows denote AGT/ANGI labeling in the same microfoci denoted in Panel A as α-SMA–positive by the white arrows. This colocalization of α-SMA and AGT/ANGI labeling is consistent with the concept that myofibroblasts in the IPF lung *in situ* constitutively synthesize AGT as they do in other organs [17] and in culture after isolation [13-16]. In Fig. (**8B**) note also the AGT/ANGI labeling of what are tentatively identified as epithelial lining cells and alveolar macrophages (arrowheads). These observations are consistent with previous demonstrations that alveolar

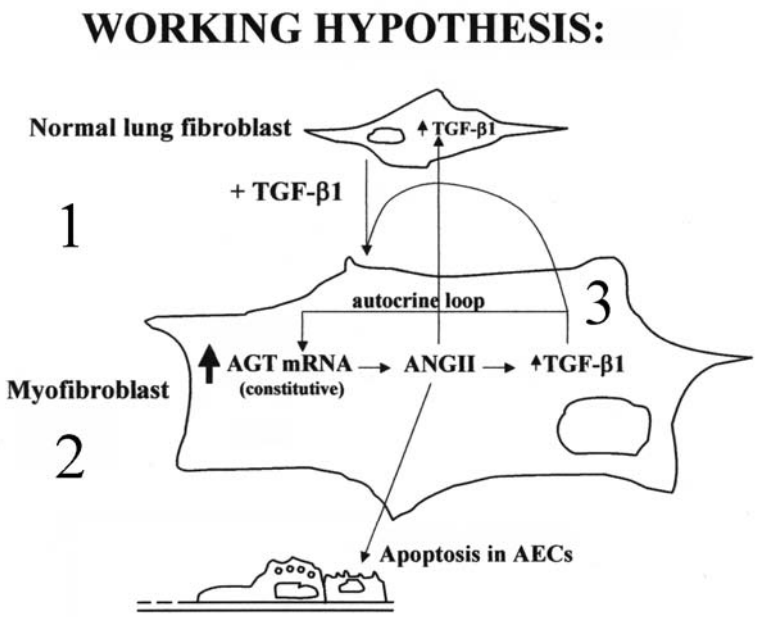

Fig. (7). Working Hypothesis for angiotensin/TGF-β1 autocrine function in human lung myofibroblasts. 1) - TGF-β1 is well known to induce the transition of normal lung fibroblasts to myofibroblasts of the VA subtype [26]. 2) - Myofibroblasts in human lung and other organs constitutively synthesize angiotensinogen (AGT) and angiotensin peptides [16] including angiotensin II (ANGII). Earlier work has shown that ANGII is an inducer of apoptosis in adjacent alveolar epithelial cells [16,23], and induces TGF-β1 synthesis in normal human lung fibroblasts [20,21]. 3) - Studies herein confirm previous work and further demonstrate that myofibroblast autocrine expression of AGT drives autocrine expression of TGF-β1, which in turn drives AGT constitutive expression to form an "autocrine loop". It is speculated that the autocrine loop recruits additional normal fibroblasts into the transition, and forms an autonomous profibrotic microenvironment that persists in the absence of inflammation, as in human IPF [25].

epithelial cells synthesize AGT while undergoing apoptosis [30,31], and with data describing ANG and macrophage function discussed below.

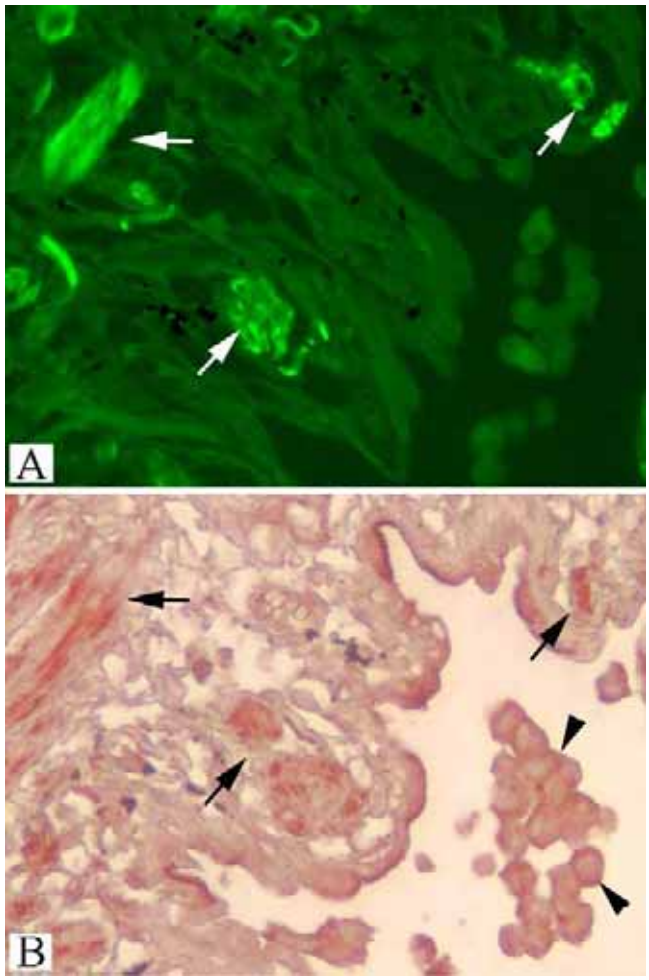

Fig. (8). Localization of AGT/ANGI immunoreactivity in myofibroblasts, epithelial cells and presumptive macrophages in human Idiopathic Pulmonary Fibrosis (IPF). Paraffin sections of a lung biopsy from a patient with IPF were immunolabeled with antibodies against alpha-smooth muscle actin (α-SMA), the myofibroblast marker (Panel A, green I= FITC direct conjugate), or with antibodies that recognize AGT and ANGI (Panel B, brown). Panels A and B are serial sections of the same microenvironment within the biopsy. In both panels, paired arrows denote identical regions in which AGT/ANGI labeling colocalizes to foci of α-SMA labeling. In Panel B, arrowheads denote AGT/ANGI labeling of presumptive alveolar macrophages. See text for details.

THE ANGIOTENSIN SYSTEM AND MONOCYTES/ MACROPHAGES

The role of the angiotensin system in modulating monocyte/macrophage functions has been largely studied in cardiovascular and renal fibrotic process in which macrophage infiltration has an important function. Some studies suggest that angiotensin II (ANGII) produced by macrophages not only affects neighboring cells such as fibroblasts, but also plays a role in macrophage function *per se* through autocrine/paracrine mechanisms [35]. It is not clear if the effects on macrophages are a direct effect of ANGII, or whether they occur through induction of other molecules [36].

A variety of *in vivo* and *in vitro* studies has demonstrated that ANGII and its degradation products are involved in the chemotaxis and adhesion of mononuclear cells in myocardial fibrosis, vascular damage and renal diseases [37-39]. A wealth of data describes ANGII-mediated production of several molecules that recruit or activate macrophages, such as cytokines (MCP-1, MIF, IL-6, TNF-α, IL-1β), endothelial cell adhesion molecules (selectins, ICAM-1 and VCAM-1) and integrins (α(v)β) [37, 40-47]. It has been shown that the expression of monocyte chemoattractant protein-1 (MCP-1), one of the most studied cytokines, is induced through ANGII receptor subtype 1a (AT1a) activation [35,37,38,48-50]. ACE inhibition and AT1 antagonism result in decreased MCP-1 levels in several animal models of cardiovascular disease and diabetes mellitus [41,51-55] and in patients with myocardial infarction [56], and the decreased MCP-1 correlated with decreased accumulation of macrophages.

There is increasing evidence that AT1 and AT2 receptors are upregulated in macrophages during their transformation from monocytes [36]. In addition, cell migration involves the degradation of basal laminae and interstitial stroma, both processes that involve matrix metalloproteinases [37]; lesional macrophages produce MMP-9 (gelatinase B) and MMP-2 (gelatinase A), whereas unstimulated macrophages constitutively secrete MMP-2 but not MMP-9. Recent findings suggest that ANGII-upregulated MMP-2 and MMP-9 expression is partially mediated by the AT1 receptor in macrophages [57]. Furthermore, transcriptional factors that could be activated by ANGII (ERK ½ and NFκB) are involved in inducible MMP-9 expression by human monocytes [37,57]. However, more studies are required to define which components of the cell migration mechanism are regulated by specific signalling pathways activated through the AT1 receptor (AT1R).

Much research has been focused on defining how ACE inhibitors and ANGII receptor antagonists modulate cellular adhesion and chemotaxis [37,40-46,58-60]. The multiple steps involving monocyte arrest, diapedesis and differentiation of macrophages are regulated by a complex interaction among different cellular responses and transcription factors. Published evidence suggests that ACE inhibitors and AT1R antagonists can modulate the transcriptional activation of cytokine genes by decreasing the concentration of ANGII available for cell signaling [61,62]. AT1 activation induces a cascade of cellular responses, including increased protein tyrosine phosphorylation, protein kinase C and intracellular calcium, all of which mediate gene transcription. Therefore, AT1 receptor upregulation in macrophages could contribute, at least in part, to induction of gene transcription *via* NF-kB and other factors such as endothelin-1 and redox-mediated pathways [41,63-65].

Oxidative stress increases the expression of the AT1 receptor in monocytes/macrophages, which can enhance macrophage formation [66]. Moreover, ANGII may induce cellular responses through free radical generation [41], particularly through NAD(P)H complex [67], which mediates the activation of protein kinase B and JAK/STAT signaling. Reactive oxygen species (ROS) mediate the induction of

monocytic chemokines and adhesion molecules through the NF-κB transcription factor [44]. NF-kB activation is multifactorial and occurs with dissociation of the inhibitory subunit IκB. ANGII appears to participate in this process through the phosphorylation of IκB, and reactive oxygen intermediates may serve as messengers in this activation [68]. Activation of NF-κB-dependent chemotactic mediators by ANGII is an additional mechanism that promotes recruitment of monocytes and the formation of macrophages [35,51,55], and ACE inhibitors and AT1R antagonist decrease NFκB activation in macrophages. Other signalling pathways involved in human monocytes migration mediated by ANGII are those involving c-Src, ERK ½, and p38, members of the MAPK family. c-Src is required for ANGII-induced phosphorylation of proline-rich tyrosine kinase 2 (Pyk2) and paxillin, two cytoskeleton-associated proteins involved in cell attachment and movement in human monocytes and other cells, and the AT1-R antagonist losartan inhibited Pyk2 and paxillin phosphorylation in monocytes [37].

Interestingly, ACE inhibitors but not AT1R antagonists prevent monocyte/macrophage infiltration in renal disease of rats, although both drugs are capable of reducing NF-κB activation. A beneficial component of ACE inhibitors may be the generation of increased nitric oxide (NO) levels [40,42]. It is well established that ACE inhibitors stimulate the release of NO from endothelium [69], which could play a protective role by restoring the redox potential, deactivating NF-κB, and consequently preventing the expression of mediators implicated in macrophage recruitment (Fig. **9**). Alternately, the effects of ACE inhibitors in this system may be due to recently described "ACE signaling" in which ACE inhibitors, binding directly to the ACE molecule, evoke ACE-mediated intracellular signaling pathways independent of ANGII action [78]. Exploring these possibilities will be interesting topics for future research.

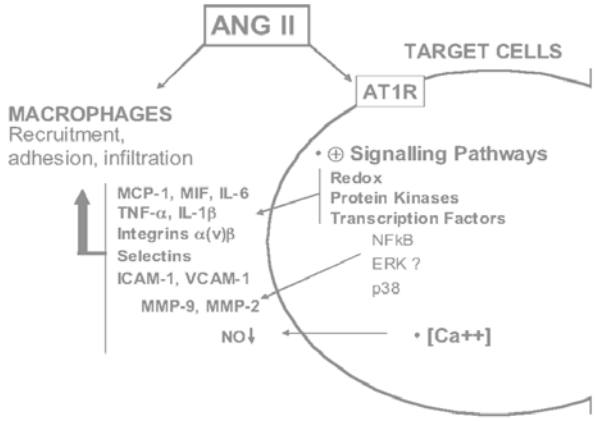

Fig. (9). Potential roles for angiotensin II in macrophages. See text for details.

ANGIOTENSIN AND MACROPHAGES IN THE LUNG

Very few data describe the role of the angiotensin system in lung alveolar macrophages. The exact role(s) of pulmonary macrophages in the pathogenesis of fibrosis is not well defined, but increasing evidence suggests that alveolar macrophages can release mediators that promote the recruitment of fibroblasts and stimulate collagen deposition. Although it is believed that AT1 receptor is expressed by lung macrophages [70], AT1 antagonist treatment in amiodarone-induced lung fibrosis has not revealed a significant reduction of interstitial macrophage infiltration [71]. There is also evidence that mononuclear cells have the ability to change their functional properties in response to different stimuli or pathological processes in the lung [72]. Earlier experiments have demonstrated that normal unstimulated alveolar macrophages and monocytes contain angiotensin peptides and an angiotensin-I-forming enzyme, suggesting that an intracellular renin-angiotensin system may exist in certain subpopulations of normal macrophages and monocytes [73,74]. Alveolar macrophages from mice and rats exhibit a fairly low, if detectable, ANGII content, at least in the resting state [73]. However, ANGII produced by macrophages and fibroblasts increases significantly in irradiated lungs [76]. Wynes *et al.* [72] observed that interstitial macrophages stimulated by IL-4 synthesize a growth factor (IGF-1) which protects myofibroblasts from apoptosis and correlates with disease severity in (IPF). Moreover, increased MCP-1 expression in IPF lung samples has been shown, compared with normal human lungs [77]. This raises the possibility that only stimulated alveolar macrophages produce ANGII in response to certain lung injuries.

CONCLUSIONS

Local renin-angiotensin systems (RAS) are now known to exist in a wide range of organs, and a variety of both pharmacologic and genetic experiments have demonstrated roles for these systems in both tissue injury and fibrogenesis. Myofibroblasts from many organs, including the human lung, contribute to the local RAS by the synthesis of AGT and ANG peptides, which in turn activate TGF-β1 expression and collagen deposition. Autocrine expression of all these molecules has been demonstrated in myofibroblasts, which are believed to be key players in matrix accumulation in the lungs, heart, kidneys, liver and other fibrosing organs. Recent studies of myofibroblasts from patients with Idiopathic Pulmonary Fibrosis demonstrate an autocrine mechanism by which TGF-β1 activates AGT and ANG peptide expression, which in turn upregulates TGF-β1 expression to form an "autocrine loop". It is hypothesized that the loop can be initiated at focal sites of injury by sustained upregulation of either ANGII or TGF-β1, and can persist in an autonomous and self-amplifying microenvironment (the "fibroblastic focus") without exogenous stimuli. In support of this view, antagonists of either molecule have been shown to block lung fibrogenesis in several different animal models and *in vitro* systems comprised of human lung cells. This theory is now being evaluated in explants of fibrotic human lung with antagonist delivery strategies designed to maximize local drug availability.

An emerging body of evidence supports a physiological role of ANGII and AT1 receptor in mononuclear cell migration and adhesion, induction of intracellular signalling and the production of several molecules involved in the recruitment and activation of macrophages. The mechanisms by which ANGII act on macrophages seem to be mediated

mainly by the AT1 receptor, but secondary mediators (ROS, NO) may also have a significant role. Interestingly, some data suggest that macrophages can produce ANGII and possibly contribute to local paracrine/autocrine mechanisms. The possibility of ANGII acting as a regulator of transcription is especially attractive because it could modulate several components of fibrotic processes. The results of cardiovascular and renal fibrosis research should be extrapolated to the lung with caution, because the action of macrophages in cardiac and renal fibrogenesis may be very different from the lung fibrotic process. Although the relative role of alveolar macrophages in lung fibrosis has not been completly elucidated, these cells could produce ANGII in certain conditions and respond by inducing the production of mediators that increase macrophage infiltration. Future research in these areas could improve the state of knowledge regarding the mechanisms by which macrophages and their actions are regulated by the angiotensin system, and vice versa.

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3. Discusión conjunta sobre el tema

Efectos del antagonismo del receptor 1 de la ANGII en la fibrosis pulmonar inducida por bleomicina en ratas

Para estudiar los efectos del antagonismo del receptor AT1 en la fibrogénesis pulmonar, se realizó un tratamiento con losartan (antagonista del receptor AT1) en el modelo experimental de fibrosis pulmonar inducida por bleomicina en ratas. En primer lugar, se realizó la estandarización y puesta a punto del modelo animal de fibrosis pulmonar. En segundo lugar, se determinó la dosis de losartan utilizada, 50 mg/kg/día, tras haber ensayado previamente dosis de 5 mg/kg/día y progresivamente superiores, y comprobar el efecto dosis-respuesta del fármaco en el modelo animal estandarizado. Finalmente, los diferentes grupos de animales de experimentación se sacrificaron a 3 y 14 días, para estudiar los cambios en las alteraciones inflamatorias y fibróticas respectivamente.

a) *Disminución de la inflamación inducida por bleomicina mediante la administración de losartan.*

Los parámetros que se utilizaron para valorar la inflamación fueron la cuantificación de mieloperoxidasa (MPO) en tejido, enzima contenida en los neutrófilos, y la determinación de las proteínas totales en el lavado broncoalveolar. Mediante estudios histológicos se evaluó tanto la lesión pulmonar como la infiltración neutrofílica.

Tras la instilación de bleomicina, a 3 y 14 días, se observó un aumento de los parámetros inflamatorios estudiados que fue inhibido significativamente mediante losartan. El efecto anti-inflamatorio al antagonizar el receptor AT1 ha sido demostrado previamente en otros modelos experimentales de fibrosis pulmonar y en estudios experimentales de enfermedades neurológicas o cardiacas en las que también existe una alteración inflamatoria (111, 126). Aunque desde hace años se conoce que la FPI no es consecuencia de una

alteración inflamatoria previa, el papel de la respuesta celular inflamatoria en la progresión de esta enfermedad sigue siendo ampliamente debatida (6, 127). En diversos modelos experimentales se ha conseguido inducir fibrosis sin una previa inflamación, y, además, el tratamiento anti-inflamatorio utilizado en los últimos años no ha demostrado mejorar el pronóstico de la enfermedad. Sin embargo, el numero total de células obtenidas del lavado broncoalveolar de los pacientes con FPI, formado básicamente por macrófagos, es claramente mayor que en sujetos sanos. Los macrófagos pueden sintetizar y secretar diferentes moléculas asociadas a la perpetuación del proceso fibrogénico. En este sentido, son escasos los datos sobre la función de los macrófagos en la fibrosis pulmonar. Al evaluar la síntesis extravascular de los péptidos de la angiotensina en tejido pulmonar de pacientes con FPI, nuestro grupo ha observado que células intra-alveolares, morfológicamente compatibles con macrófagos, expresaban ANGEN, mientras que no lo hacían en tejido pulmonar de sujetos normales. Estas observaciones sugieren que los macrófagos podrían realizar funciones esenciales en la progresión de la FPI. Los resultados presentados, sumados a los de otros trabajos que abogan por la importancia de diferentes mediadores secretados por los macrófagos en la fisiopatología de la FPI (128), hacen necesaria la realización de nuevos estudios que evalúen la función exacta de estas células en la enfermedad.

b) El tratamiento con losartan inhibe el depósito de colágeno inducido por bleomicina.

Para estudiar la formación de la matriz extracelular se determinó la cantidad de hidroxiprolina en el pulmón, medida indirecta del contenido de colágeno, y se evaluaron las áreas de fibrosis pulmonar en preparaciones histológicas mediante la tinción con tricrómico de Masson. Se observó un aumento significativo del contenido de hidroxiprolina tras la inducción con bleomicina. Este incremento fue inhibido en el grupo que recibió tratamiento con losartan. Estos resultados concuerdan con estudios previos realizados con otros

antagonistas del receptor AT1 (111) y en otros modelos animales de fibrosis pulmonar (129). Este efecto podría ser debido a la inhibición de las señales intracelulares activadas con la unión de la ANGII al receptor AT1 que favorecen, directa o indirectamente, la formación del depósito de colágeno.

c) *El efecto antifibrótico del antagonismo del receptor AT1 mediante losartan se debe, en parte, al incremento en la cantidad de prostaglandina E2.*

La PGE2 es un mediador esencial para la correcta reparación del epitelio alveolar lesionado en la fibrosis pulmonar, inhibe la migración y proliferación fibroblástica, y disminuye el depósito de colágeno que induce el TGF-β1 (130-132). Diversos estudios demuestran que la síntesis de PGE2 está disminuida en fibroblastos de pulmones con FPI (74). Los resultados del presente estudio demuestran que la fibrosis pulmonar inducida por bleomicina provoca una disminución en la síntesis de PGE2, lo que se evita con el antagonismo del receptor AT1. Este efecto se asocia a una reducción del depósito de colágeno, lo que aumenta los fundamentos para considerar a la PGE2 una molécula protectora en el proceso fibrótico pulmonar. Asimismo, estos resultados sugieren que la ANGII puede regular, no sólo la síntesis de TGF-β1 como previamente se había observado (99), sino también la producción de PGE2. El antagonismo del receptor AT1 podría disminuir la síntesis de TGF-β1 y aumentar la producción de PGE2, favoreciendo la homeostasis entre factores de crecimiento pro-fibróticos y anti-fibróticos e inhibir así la progresión del proceso fibrogénico pulmonar. Quedan por aclarar dos aspectos relacionados con estas observaciones y que se abordan actualmente:

1) comprobar que este efecto modulador del antagonismo del receptor AT1 también se observa en tejido pulmonar humano,

2) investigar los mecanismos mediante los cuales el antagonismo del receptor AT1 regula la síntesis de la PGE2. La ciclooxigenasa-2 (COX-2) es la enzima principal que sintetiza PGE2 y su actividad se ha encontrado

disminuida en fibroblastos de pacientes con FPI (133). En el presente estudio el antagonismo del receptor AT1 aumenta la expresión de COX-2 a los 3 días, pero no a los 14 días. Por otro lado, no se observa una correlación entre el incremento en la cantidad de PGE2 y la expresión de RNAm de COX-2. Teniendo en cuenta las dificultades al medir la actividad de esta enzima, una explicación para estos hallazgos podría ser la existencia de otras fuentes de producción de PGE-2 modificables al antagonizar el receptor AT1, como la PGE2 sintetasa.

Relevancia del efecto de los péptidos de la angiotensina en la fisiopatología de la FPI

Los resultados presentados en el primer artículo afianzan las observaciones previas realizadas por el grupo de Uhal y colaboradores en las que se demuestra la importancia del efecto de la ANGII en la fisiopatología de la fibrosis pulmonar experimental y añaden además un nuevo mecanismo mediante el cual la ANGII puede actuar favoreciendo la fibrogénesis pulmonar al inducir la disminución de la PGE2. Para valorar si los hallazgos observados en los modelos experimentales de fibrosis pulmonar podrían ser relevantes en la FPI, se investigó el impacto del sistema angiotensina en tejido pulmonar de pacientes con FPI comparado con tejido pulmonar normal mediante estudios inmunohistoquimicos, rt-PCR y Western-blot.

a) *En la FPI existe un aumento en la síntesis local de los péptidos de la angiotensina.*

El análisis de preparaciones histológicas para inmunohistoquímica y de tejido pulmonar fresco para rt-PCR y western-blot mostró que la cantidad de ANGEN y ANGII es significativamente mayor en los pulmones de pacientes con FPI. Los estudios previos que investigaron la posible síntesis “local” de los péptidos de la angiotensina fueron realizados en fibroblastos y células epiteliales cultivadas, y objetivaron la capacidad de estas células para sintetizar ANGII (88,91). Los resultados del presente estudio demuestran que

la síntesis local de los péptidos de angiotensina está incrementada en el pulmón de pacientes con FPI y, por lo tanto, que la ANGII se encuentra claramente implicada en la fisiopatología de esta enfermedad.

b) Células pulmonares extravasculares capaces de sintetizar péptidos de la angiotensina: miofibroblastos, células epiteliales alveolares y macrófagos

En las preparaciones histológicas de pulmones sanos, las células que captan la señal para el anticuerpo de ANGEN son únicamente las células vasculares y las células musculares peribronquiales. Sin embargo, en las preparaciones de tejido pulmonar obtenido de pacientes con FPI, las células en las que se observa proteína de ANGEN son células epiteliales alveolares, principalmente apoptóticas, miofibroblastos y células intraalveolares morfológicamente compatibles con macrófagos. La implicación de estas células se corroboró mediante doble marcaje inmunohistoquímico, con anticuerpos específicos para células epiteliales (anticuerpo Mab MNF-116, o anti-citoqueratina), células epiteliales apoptóticas (marcaje in situ del DNA fragmentado, *ISEL*), y para miofibroblastos (anticuerpo anti-α-smooth muscle). Se observó que células intraalveolares compatibles con macrófagos captaban la señal para ANGEN. Dado que no fue posible utilizar un marcaje específico fenotípico para estas células en las muestras estudiadas, se evaluó la posibilidad de ser células epiteliales mediante marcaje inmunohistoquímico con MNF-116, para lo que fueron negativas. Actualmente nuestro grupo ha iniciado estudios para corroborar y evaluar la implicación de los macrófagos en la síntesis de péptidos de la angiotensina. Los resultados demuestran que, a parte del aumento cuantitativo de los péptidos de la angiotensina en la FPI, existe una alteración en la función celular ya que se ha objetivado un aumento proteico de ANGEN en células epiteliales alveolares alteradas, miofibroblastos, y, probablemente, macrófagos. La importancia de la alteración de células epiteliales y miofibroblastos en la fisiopatología de la FPI ha sido demostrada

ampliamente, tal como se ha descrito en la introducción, si bien la implicación de los macrófagos es un tema que sigue en estudio actualmente.

4. Conclusiones

1. La síntesis de ANGII está aumentada en la FPI y es un potente mediador fibrogénico implicado en la fisiopatología de la fibrosis pulmonar.

2. En la FPI se identifican células extravasculares capaces de sintetizar ANGEN y ANGII: células epiteliales alveolares, principalmente apoptóticas, miofibroblastos y macrófagos.

3. El antagonismo del receptor AT1 mediante losartan disminuye la fibrosis pulmonar inducida por bleomicina en ratas. La administración de losartan inhibe el depósito de colágeno e interfiere en el descenso de PGE2 que induce la bleomicina. Por lo tanto, el efecto antifibrótico de losartan puede deberse no sólo a la inhibición de las acciones mediadas por la ANGII, sino también al favorecer la síntesis de PGE2, molécula con acciones antifibrogénicas.

4. Dada la implicación de los péptidos de la angiotensina en la FPI y el efecto antifibrogénico del antagonismo del receptor AT1 en la fibrosis pulmonar experimental, resulta evidente que la utilización de los antagonistas del receptor AT1 podría incluirse en futuras estrategias terapéuticas a aplicar en pacientes con FPI.

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