胞が壊れたときにも出血しやすくなるのが特徴です。これをDIC(播種性血管内凝固症候群)といいます。この原因としては、急性前骨髄球性白血病細胞から放出されるプラスミノーゲンアクチベーターにより過度のプラスミンの産生、フィブリンの分解、蛋白分解酵素の活性化が起こること、治療によって細胞死を起こした白血病細胞からトロンビンの産生増加が亢進していることなどが考えられています。

4)急性前骨髄球性白血病が疑われたら

この病気は、他のタイプの白血病に比べて白血球数が減少することが多く、感染症を合併しやすくなります。

また、血小板減少だけでなく、凝固を促進する物質の増加によって体内で出血しやすくなり、脳出血や消化管出血が主な原因となって亡くなることもあります。そのため、できるだけ初期のうちに発見することが大切です。

5)どのような検査をするか

この病気は、全身性疾患なので、体のあらゆる部位について詳細に理学的な観察が行われます。特に出血したところ(たとえば、皮膚、口腔内、歯肉、鼻の出血の有無、または採血部位などの斑状出血、点状出血)に注意が必要です。確定診断のためには、出血傾向に関する血液検査、末梢血液検査と骨髄穿刺検査の他に、末梢血や骨髄の材料を用いた染色体検査や表面マーカー検査、遺伝子検査を行うなど、さらに詳細な検査が必要です(表1)。

骨髄穿刺検査

他のタイプの白血病よりも白血球数が少ない場合や、血小板数の割に出血傾向が強い場合は、慎重に行います。他のタイプに比べると、出血が止まりにくいために血腫ができやすく、また、ドライタップといって、検査の際に骨髄細胞を取りにくいとか、凝固しやすいという特徴があります。その他、経過中に線維化といって、骨髄細胞を取りにくくなることもあります。それに、細胞形態が特徴的であるために、すぐに病気を疑うことができます。

凝固検査

発症時の白血球は正常から低値のことが多く、ほとんどの患者でDICを合併しています。これは、芽球が30%以下で、前骨髄球が増加し、血小板数が減少していることに起因しています。凝固検査では、血小板数、フィブリン、D-ダイマーなどを用いて、病気が悪化しているのか改善しているのかを判定することができます。

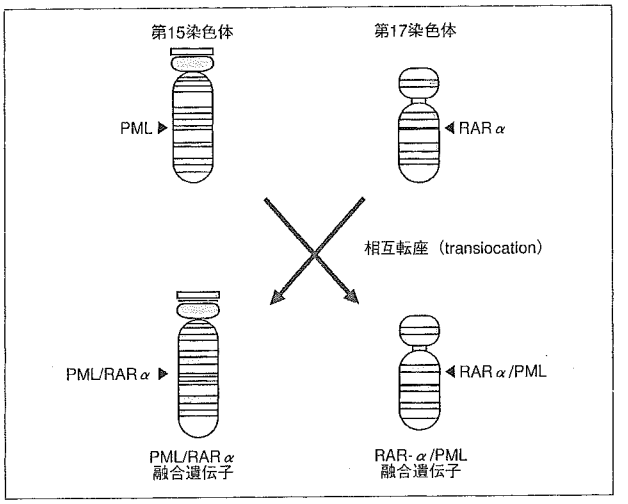
6)急性前骨髄球性白血病のタイプ

染色体異常と遺伝子検査がなぜ必要なのかについて

現在、この病気の約80%で染色体(15;17)に転座があることが明らかになっています。第15染色体(15q22)に位置するPML遺伝子と、第17染色体に位置するRARα遺伝子の相互転座によりPML/RARαキメラ遺伝子が形成され、PML/RAR

【表1】 検査項目

検査項目
血液検査
白血球数、白血球分画、ヘモグロビン値、血小板数、網状赤血球、白血球分画(血液像)、GOT(AST)、GPT(ALT)、ビリルビン、LDH(乳酸脱水素酵素)、コリンエステラーゼ、BUN(血中尿素窒素)、総コレステロール、血糖、尿酸、成人T細胞性白血病ウイルス(HTLV1)、AIDSウイルス(HIV)
骨髄穿刺検査
有核細胞数、網状赤血球、巨核球数、骨髄像、特殊染色(ペルオキシダーゼ、鉄、PAS、エステラーゼ)、染色体分析、表面マーカー
凝固検査
血小板数、フィブリン、D-ダイマー、PT、ARTT、FOP
その他の検査
心電図、胸部レントゲン、検尿、遺伝子検査



【図2】 急性前骨髄球性白血病の染色体異常
約80%の症例で染色体転座t(15;17)が認められる。
この転座により、PML-RARα融合(キメラ)遺伝子が発現している。
PML遺伝子:第15染色体上に存在する転写関連遺伝子
RARα遺伝子:第17染色体上に存在するレチノイン酸受容体α鎖
(RARα:retinoic acid receptor-α)

α遺伝子産生蛋白がこの病気の病態と深く関わっていると報告されています(図2、表2)。また、染色体検査と遺伝子検査を一緒に行うことによって再発しやすいかどうかを調べることができます。

7)急性前骨髄球性白血病の治療

急性前骨髄球性白血病の治療では、ベサノイド《All-trans retinoic acid(ATRA):トレチノイン(商品名:ベサノイド)》による寛解導入療法が、まず最初に行われます(図3)。

寛解導入療法では、ベサノイド45mg/m²を毎日3回に分けて食後に服用します。朝食後の服用を最も多くして、たとえば、1日7錠(70mg)の場合は、朝4錠、昼2錠、夜1錠服用します。白血球3,000/μLかつ急性前骨髄球性白血病細胞(芽球+前骨髄球)<1,000/μL以下の場合はベサノイド単独服用で、有効な場合は1~2週間で出血傾向が改善します。

化学療法との併用はどういうときに行うのか?

白血球3,000/μL以上では、ベサノイドとアントラサイクリン系とシタラビンを併用投与します。ベサノイド療法開始後、ベサノイド単独では白血球が増多しやすいので、頻回に採血検査を行い、白血球の増多が認められる場合はアントラサイクリン系とシタラビンの投与を併用します。

【表2】 急性骨髄球性白血病における染色体異常とキメラ遺伝子

染色体異常	融合遺伝子	ATRAに対する反応性
t(15;17) q(22;q21)	*PML/RARα*	あり
t(5;17) q(35;q21)	*NPM/RARα*	あり
t(11;17) q(13;q21)	*NuMA/RARα*	あり
t(11;17) q(23;q21)	*PLZF/RARα*	なし
duplication of (17q21.3-q23)	*STAT5b/RARα*	なし

地固め療法と維持療法について

骨髄穿刺検査で完全寛解に到達後、28日間内服を継続し、地固め療法開始前日まで内服します。平均内服期間は45日間です。ベサノイドと化学療法を併用した場合の予後が極めてよく、その後の維持療法にもベサノイドを導入した場合は、再発例が少なかったとの報告がありますので、最近では、むしろ積極的に服用したほうがいいとされています。

日本では、寛解を得られた後は、アントラサイクリンとシタラビンを組合せた他剤併用療法による地固め療法、維持療法を行います。近年の報告では、ベサノイドとダウノルビシンを寛解導入療法に用いて、強化療法にベサノイド内服を行い、維持療法にもベサノイドを用いた場合に5年生存率74%を得られ、維持療法を行わなかった場合でも5年生存率55%を得られたという報告もあります。

造血幹細胞移植

この病気では、積極的に移植を奨めてはいませんが、再発が繰り返された場合には考慮します。

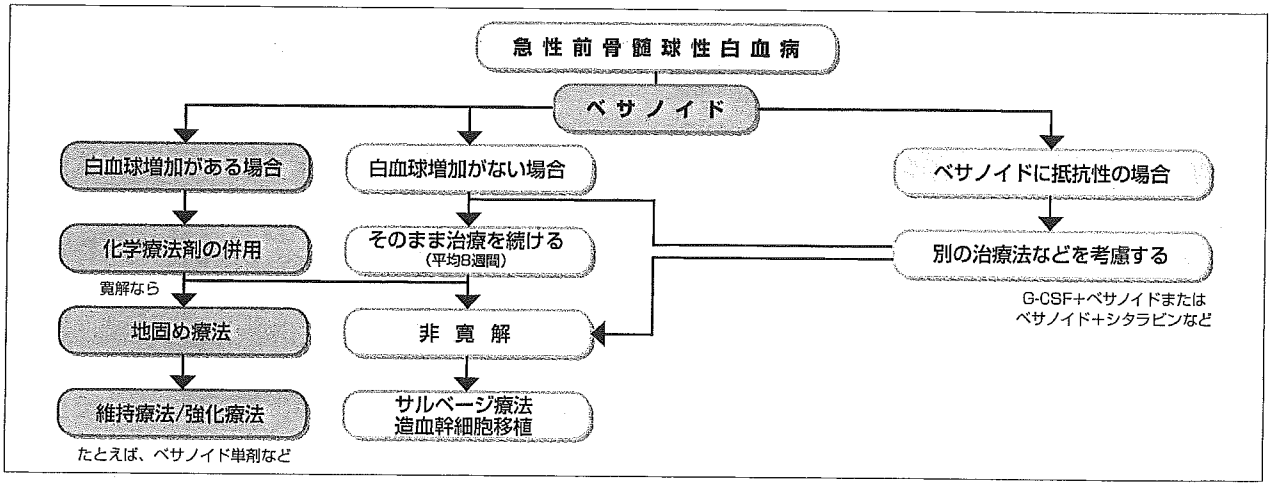
8)予後因子

急性前骨髄球性白血病では、ベサノイドによる寛解導入療法後の寛解率は80~90%、5年生存率40~50%以上と良好な成績が得られています。一般には、発病してすぐに脳出血などの出血による死亡がありますが、適切に早く治療され、かつ効果が出れば、むしろ他のタイプの白血病よりも予後がよいとされています。

9)再発

ベサノイドの再投与

最近では、ベサノイドだけで治療することは少ないですが、以前に治療を受けていて再発した場合は、ベサノイドの再投



【図3】 急性前骨髄球性白血病の治療方針

与によって有効となる場合があります。しかし、有効でない場合は、化学療法としてシタラビンのみを併用し、それにG-CSFを少量併用することがあります。どちらにしても、すでに化学療法剤が投与されている場合は、心臓機能などの検査も受けてから投与を検討します。

10)治療の副作用

ベサノイドの副作用

ベサノイドの副作用としては、一般的に皮膚や口唇の乾燥、胃腸障害、高脂血症、肝障害などの副作用の他にレチノイン酸症候群が有名です(表3、表4)。急激な白血球の増多とともに、発熱、むくみ、呼吸不全、間質性肺炎、胸水、心のう液貯留、急性腎不全などがみられます。これらの症状は、必ずしも白血球増多の時期と一致するとは限りませんが、治療には、ステロイド投与が非常に有効です。

また、稀な副作用としては内分泌代謝異常、骨髄の線維化、血栓傾向、皮膚疾患などが報告されています。これらの副作用が現れた時は、症状が重篤でなければステロイドを併用し、ベサノイド内服を継続すべきです。また、重篤な症状が出現した場合は、一旦ベサノイドを中止し、ステロイドなどで症状が軽快した後に、注意しながらベサノイドを少量から内服開始します。

また、近年ではベサノイド療法後の再発症例に対して、レチノイン酸誘導体であるAm80やヒ素の有効性が報告されていますが、日本では現在導入にいたっておりません。

【表3】 ベサノイドの副作用

●主な副作用	
・ トリグリセライド上昇	(約20%)
・ AST (GOT) 上昇	(約10%)
・ ALT (GPT) 上昇	(約10%)
・ 口唇乾燥	(約10%)
・ 頭痛	(約7%)
・ 発熱 (レチノイン酸症候群の随伴症状)	(約7%)
●重大な副作用	
・ レチノイン酸症候群	
・ 血管炎	
・ 白血球増多症	
・ 感染症(肺炎、肺血症など)	
・ 血栓症(脳梗塞、肺梗塞、その他の動脈または静脈血栓症など)	
・ 錯乱	

― ベサノイド使用に関する注意事項 ―

- 次のような方は、使用前に主治医に相談しましょう。
  妊婦または妊娠している可能性がある方。肝機能、腎機能が低下している方。高中性脂肪血症の方。以前に薬を飲んで発疹が出たことがある方。また、他に使用している薬がある場合は、主治医にお伝えください。
- この薬は、必ず指示に従い、水またはぬるま湯と一緒にお飲みください。飲み忘れた場合は、次の服用時間に1回分をお飲みください。2回分を1度に飲むことは絶対にお止めください。
  誤って多く飲んだ場合は、すぐに主治医にご相談ください。また、25歳以下の方、特に幼児および小児は、主治医の指示を必ずお守りください。また、生活上の注意としては、女性の方は、投与期間中および投与中止後少なくとも1カ月間は避妊することが大切です。
- この薬は、飲む人によっては目的以外の望ましくない作用が出ることがあります。次のような症状(発熱、胸苦しさ、空咳、息切れ、呼吸困難、立ちくらみ、全身倦怠感など)に気づいたらすぐに主治医にご連絡ください。また、次のような症状(皮膚・唇の乾燥、発疹、頭痛、悪心・嘔吐、骨・筋の痛みや運動障害など)で耐えられない場合は、なるべく早く主治医にご相談ください。
- 他にも何か変だと感じたら、主治医または薬剤師にご相談ください。

【表4】 レチノイン酸症候群

(1)レチノイン酸症候群の特徴

症　状: 発熱、呼吸困難、胸水貯留、肺浸潤、間質性肺炎、肺うっ血、心のう液貯留、低酸素血症、低血圧、肝不全、腎不全、多臓器不全など

臨床検査: 低酸素血症、末梢血白血球数の急激な増加など

(2)注意を要する臨床所見

初期症状として発熱が認められることがあるので、その場合には肺に関する検査等の実施を検討します。

成熟顆粒球の増加時に息切れなどの呼吸器症状が発現した場合は、直ちにベサノイドの服用を中止し、酸素飽和度の測定、胸部X線撮影、胸部CTスキャン、血液ガス分析を行い、以上の検査で異常所見が認められた場合は、レチノイン酸症候群の可能性があるため、副腎皮質ホルモン剤によるパルス療法や、人工呼吸器による管理、利尿剤の投与等の適切な処置を行いましょう。

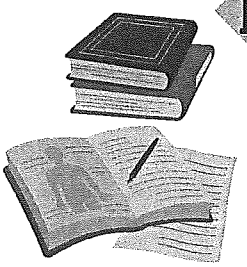
また、息切れなどの呼吸器症状がなくとも、投与後3～14日目に定期的に呼吸器所見を検討することが大切です。

(3)レチノイン酸症候群に対する治療

・ベサノイド投与中止
・副腎皮質ホルモン剤によるパルス療法
・人工呼吸器による管理

(4)催奇形性について

ベサノイドには催奇形性がありますので、妊婦または妊娠している可能性がある婦人は服用しないようにしましょう。また、妊娠する可能性がある婦人は服用しないことが原則ですが、やむを得ず服用する場合は、必ず主治医に相談しましょう。



血液よもやま話

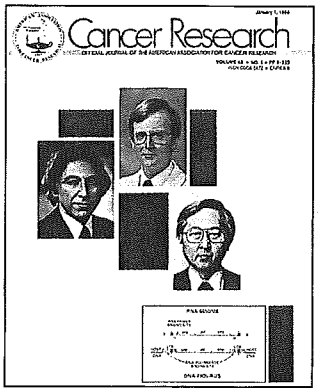
ATL発見から4半世紀

（財）田附興風会医学研究所北野病院理事
熊本大学名誉教授
高月　清
Kiyoshi Takatsuki

今さら回顧談でもあるまいが、研究の年代記と若干の感想を書きとどめる。成人T細胞白血病・リンパ腫（Adult T-cell Leukemia/Lymphoma：ATL）が、日本で発見・記載されたことは、つくづくよかったと思う。なぜならば、病原体の発見が先行すれば、多数の患者を抱えていた日本の血液学の面目はまるつぶれになって、おそらく、日本は米国の草刈場となり、研究も後塵を拝するのみとなっていたであろう。しかしながら、この分野に関する限り、日本がリードしてきたと言える。

白血病の教科書である「Henderson, Lister & Greaves：Leukemia」の第7版（Saunders、2002）においてもATLは1章が充てられ、われわれ（松岡・高月）が続けて執筆した。また、1994年には私が筆者となって、内外の研究者とともに単行書「Adult T-cell Leukemia」をOxfordから出版した。

今にして思えば、私がATLの研究を始めたのは、京都大学で助手から講師になった1972年頃である。「新しい白血病」として日本癌学会総会で示説発表したのが1975年、国際血液学会で報告したのが1976年、Blood誌に論文が掲載されたのが1977年である。



【図】 Cancer Research: Vol.48, No.1（1988年1月1日）の表紙。
William A Blattner（疫学）、Robert C Gallo（ウイルス）、高月清（臨床）

私は、もともと多発性骨髄腫と類縁疾患に興味があったが、免疫学の進歩により、研究の幅を広げてリンパ性白血病のT・B分類を始めた。そして、すぐに気づいたことは、日本人の成人では欧米の報告と異なり、T細胞性の白血病が意外に多いということであった。さらに、症例を重ねて分かったことは、T細胞性白血病の患者の出身地が九州に偏っていることであった。初めは冗談のように言っていたが、例数の増加とともに確信に変わり、また臨床上の特徴も明らかになり、「成人のT細胞白血病」と名づけて報告した。

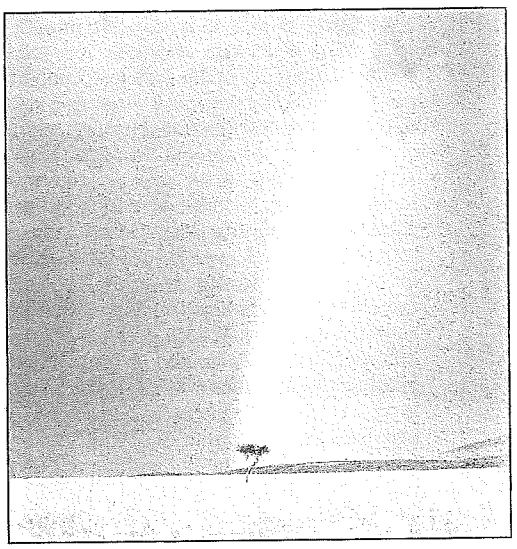
成人のT細胞白血病の病因にレトロウイルス（今ではHTLV-1と呼ばれる）が関与することが分かったのは1981年で、ウイルスの発見者は、米国のRobert Gallo先生と日本の日沼頼夫先生であった。これは、三好勇夫先生が培養細胞MT-1を得て、日沼先生が間接蛍光抗体法によって患者血清がMT-1細胞に反応することを見出し、ウイルスが分離されたのである。そして、吉田光昭先生は、日米で別々に発見されたウイルスが同じ構造を持つことを証明した。米国でHTLV-1が分離された2人の患者の病名は、それぞれ菌状息肉症（1980年）とSezary症候群（1981年）で、ともにATLであったことは明らかである。

その後、私は熊本大学に転じ、14年余りの間多くの共同研究者とともに研究を展開することができた。

これまで述べてきた研究などが実を結び、2002年には、京都で癌学会シンポジウム「ウイルス発見20周年」が開催されたほか、国際的にもヒトレトロウイルスHTLV関連国際会議（第5回：1992年・熊本、第11回：2003年・サンフランシスコ）が開催されるようになった。

昨年の11月に、HTLV-1の発見者であるGallo先生が日本エイズ学会で来日した折に、病院に私を訪ねて来られたが、ATLとHTLV-1がエイズとHIVの研究に貢献したことは言うまでもない。

表紙photo



ケニヤの首都ナイロビから南西に約260キロ（車で5時間ほど）のところにあるマサイマラ国立保護区は、面積が1,500平方kmを超える雄大さで、国境を接するタンザニアのセレンゲティ国立公園とともに一大生態系を形成し、まさに「野生の王国」と言える。
見渡す限りのサバンナ（大草原）だけでなく、森や湿地帯、丘陵など様々な色合いと変化に富んだ地形が、多くの野生動物たちの住み良い環境をもたらしている。サバンナの乾期が終わる頃、小雨季の到来を伝える雨が多くなる。雨雲の切れ間から、サバンナの一本木に木漏れ日がスポットライトのように当たり、地平線の中に大きな虹が現われた。

撮影者：　飯島正広
タイトル：　サバンナの虹
提　　供：　ネイチャー・プロダクション

●発行：オーシーシー・ジャパン株式会社

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PANCREAS

Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis

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Background and aim: The present study was conducted to examine the effect of activin A on activation of rat pancreatic stellate cells (PSCs).
Methods: PSCs were prepared from rat pancreas using collagenase digestion and centrifugation with Nycodenz gradient. Activation of PSCs was examined by determining smooth muscle actin expression with western blotting. The presence of activin A receptors in PSCs was investigated by reverse transcription-polymerase chain reaction (RT-PCR), western blotting, and immunocytochemistry. Expression of activin A and transforming growth factor β (TGF-β) mRNA was examined by RT-PCR. Activin A and TGF-β peptide concentrations were examined with ELISA. Existence of activin A peptide in PSCs was investigated by immunocytochemistry. Collagen secretion was determined by Sirius red dye binding.
Results: Activin A receptors I and IIa were present in PSCs. PSCs expressed activin A mRNA and secreted activin A. Activin A enhanced PSC activation and collagen secretion in a dose dependent manner. TGF-β and activin A increased each other's secretion and mRNA expression of PSCs. Follistatin decreased TGF-β mRNA expression and TGF-β secretion of PSCs, and inhibited both PSC activation and collagen secretion.
Conclusion: Activin A is an autocrine activator of PSCs. Follistatin can inhibit PSC activation and collagen secretion by blocking autocrined activin A and decreasing TGF-β expression and secretion of PSCs.

Activin A is a homodimeric protein with a molecular mass of 25 kDa, originally isolated from ovarian fluid as a stimulant for follicle stimulating hormone secretion.[1] Based on its molecular structure, activin A is considered to be a member of the transforming growth factor β (TGF-β) superfamily.[2] Subsequent studies have revealed diverse actions of activin A on cellular growth and differentiation in various types of cells. Activin A increases the number of gonadotrophs in anterior pituitary cultures[3] and modulates differentiation of ovarian granulosa cells.[4] Activin A also induces differentiation of erythroid cells[5] and megakaryocytes,[6] and inhibits hepatocyte proliferation.[7] In the pancreas, activin A is present in islets A and D cells[8] and regulates B cell differentiation.[9] Follistatin is an endogenous activin A binding protein known to block the effect of activin A.[10] For instance, follistatin protected the activin inhibitory action on hepatocyte proliferation in vitro,[7] and overexpression of follistatin in epidermis of transgenic mice impaired wound healing which is controlled by activin A.[11] Thus follistatin can be used as a potent activin A blocker both in vitro and in vivo.

Pancreatic stellate cells (PSCs) have been recently identified, isolated, and characterised.[12 13] In the normal pancreas, PSCs possess fat droplets containing vitamin A and are quiescent, defined by desmin positive but α smooth muscle actin (α-SMA) negative staining.[12] When cultured in vitro, PSCs are autoactivated (autotransformed) changing their morphological and functional features.[13] PSCs commence losing vitamin A containing lipid droplets, are highly proliferative, with increasing expression of α-SMA, and produce and secrete extracellular matrix components such as collagen and fibronectin. Namely, PSCs are autotransformed to myofibroblast-like cells. In vivo, PSCs are also activated during both human and experimental pancreatic fibrosis.[14] Therefore, PSCs are believed to play an important role in pancreatic fibrogenesis.

TGF-β is one of the major profibrogenic cytokines in various tissues. Recently, evidence of TGF-β participation in pancreatic fibrogenesis has been found. Transgenic mice overexpressing TGF-β in islet cells develop fibrosis of the exocrine pancreas. Moreover, inhibition of TGF-β by anti-TGF-β antibody reduced extracellular matrix production in rat cerulein pancreatitis.[15] TGF-β has also been shown to promote PSC activation and extracellular matrix production in an autocrine manner. TGF-β secreted from cultured PSCs controls PSC growth and promotes their activation and extracellular matrix production.[16 17] In human chronic pancreatitis tissue, TGF-β expression was observed in acinar cells adjacent to areas of fibrosis and in spindle cells in fibrotic bands.[14] Thus TGF-β is assumed to promote pancreatic fibrosis, in part by activating PSCs and enhancing their extracellular matrix production. Although activin A is also a member of the TGF-β family and its physiological role in pancreatic cellular differentiation has been well described,[9 18] its pathophysiological role in pancreatic disorders is still unknown. As activin A modulates cell growth and differentiation of various cells in a variety of organs, it is an interesting possibility that activin A also modifies PSC function and participates in pancreatic fibrosis.

In the present study, we examined the effect of activin A on PSC function. We report here that activin A activates PSCs and collagen secretion. Furthermore, activin A is released from PSCs, and activin A and TGF-β enhance each other's secretion and mRNA expression of PSCs. Finally, follistatin blocks PSC activation and collagen secretion by blocking

Abbreviations: PSC, pancreatic stellate cell; HSC, hepatic stellate cell; α-SMA, α smooth muscle actin; TGF-β, transforming growth factor β; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

activin A and reducing TGF-β release and mRNA expression of PSCs. Hence activin A is an autocrine activator of PSCs and follistatin can be used potentially in the treatment of pancreatic fibrosis.

METHODS

Materials

Recombinant human activin A and rabbit antiactivin A polyclonal antibody were kindly provided by Dr Yuzuru Eto (Ajinomoto Co., Japan). Pronase, Nycodenz, and anti-SMA antibody were purchased from Sigma (St Louis, Missouri, USA). DNase I was from Roche (Basel, Switzerland). Collagenase P was from Boehringer Mannheim (Mannheim, Germany). Follistatin was from R&D Systems (Abington, UK). Antiactivin receptor type I and type IIa antibodies were from Santa Cruz (Santa Cruz, California, USA). Horseradish conjugated donkey antimouse IgG, Cy3 conjugated donkey antirabbit IgG, Cy3 conjugated donkey antigoat IgG, and FITC conjugated donkey antimouse IgG antibodies were from Jackson Immuno Research (West Grove, Pennsylvania, USA).

Isolation and culture of rat pancreatic stellate cells

Rat pancreatic stellate cells were prepared as described previously.[12] Briefly, rat pancreas was digested in Gey's balanced salt solution supplemented with 0.05% collagenase P, 0.02% pronase, and 0.1% DNase I. After filtration through nylon mesh, cells were centrifuged in a 13.2% Nycodenz gradient at 1400 *g* for 20 minutes. PSCs in the band just above the interface of the Nycodenz solution and the aqueous one were collected, washed, and resuspended in Iscove's modified Dulbecco's medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. PSCs were cultured in a 5% CO_2 atmosphere at 37°C. All experiments were carried out using PSCs between passages one and two.

Western blotting

Western blotting was carried out as described previously,[19] using the enhanced chemiluminescence reagent to visualise the secondary antibody. For gel electrophoresis, 10 μg of protein were loaded on each lane of a 10% sodium dodecyl sulphate-polyacrylamide gel.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously[20] using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan). Images were digitised and then processed using Photoshop 5.0 software (Adobe Systems Inc., Mountain View, California, USA).

Quantification of activin A and TGF-β

Concentrations of activin A and TGF-β in culture media of PSCs were determined using commercial kits from R&D Systems and DRG International (Mountainside, New Jersey, USA), respectively, according to the manufacturer's instructions.

Quantification of collagen secretion

Collagen secreted into culture medium by PSCs was determined using Sircol Sirius red dye (Biocolor Ltd, Newtownabbey, UK), as described previously.[21] Collagen was measured by spectrophotometry at 540 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from PSCs with TRIzol reagent (Life Technologies BRL, Grand Island, New York, USA). First strand cDNA was made from total RNA using ReverTra Ace system (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. PCR for TGF-β was carried out using the PCR kit for rat TGF-β (Maximbio, San Francisco, California, USA). PCR for rat activin A β_A subunit, rat activin type I receptor, rat activin type IIa receptor, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with the following primers:

- rat activin A β_A subunit: sense 5′-GGACCTAACTCT-CAGCCAGAGATG-3′, antisense 5′-TCTCAAA-ATG-CAGTGTCTTCCTGG-3′;
- rat activin type I receptor: sense 5′-GGTCTAT-GAGCAGG-GGAAGATGAC-3′, antisense 5′-ACATTTTCGCCTTGCC-AGC-3′;
- rat activin type IIa receptor: sense 5′-AGATGGAAG-TCACACAGCCCAC-3′, antisense 5′-CAACACTGGT-GCCTCTTTTCTCTG-3′;
- rat GAPDH: sense 5′-CATGACCACAGTCCATGCCATC-3′, antisense 5′-CACCCTGTTGCTGTA-GCCATATTC-3′.

The reactions were conducted in a DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, Connecticut, USA) with the following cycle conditions: denaturation at 94°C for one minute, annealing at 52°C for 45 seconds, and extension at 72°C for 45 seconds. The number of cycles was 30 for rat activin A β_A subunit, TGF-β, and rat activin receptors, and 18 for GAPDH.

Statistical analysis

ANOVA was used to determine statistical significance. A p value of <0.05 was considered significant.

RESULTS

Activin receptors I and IIa are present in PSCs

As the first attempt to elucidate activin A effects on PSCs, we examined the presence of activin A receptors in PSCs. As activin receptors function as a heterodimer of type I and type II receptors, we examined the presence of both activin receptor type I and type IIa. As shown in fig 1A, RT-PCR with primers specific for rat activin receptor I and IIa amplified 480 (activin receptor I) and 423 (activin receptor IIa) base pair sequences, respectively. These PCR products were verified by sequencing (data not shown). These data indicate that mRNAs of both activin receptors I and IIa are expressed in PSCs. To confirm expression of activin receptor I and IIa proteins, we carried out western blotting of PSC lysate using antiactivin receptor I and IIa antibodies. As shown in fig 1B, western blotting revealed expression of both activin receptor I and IIa proteins in PSCs. As an independent test of the presence of activin receptors, we applied immunocytochemistry using antiactivin receptor I and IIa antibodies. As shown in fig 2, immunocytochemistry revealed that both signals of activin type I and type IIa receptors are observed in PSCs. These signals were abolished when antibodies were pre-incubated with competing peptides to each antibody (data not shown). These data indicate that activin receptor components essential in forming functional activin receptor heterodimers are present in PSCs.

Activin A activates PSCs and increased collagen secretion

We next examined the effect of activin A on PSC activation and collagen secretion. As shown in fig 3A, activin A increased the amounts of α-SMA in PSCs in a dose dependent manner. The maximum increase was observed at 1 nM. Activin A also enhanced collagen secretion by PSCs (fig 3B). These data indicate that activin A activates PSCs and suggest activin A participation in pancreatic fibrosis.

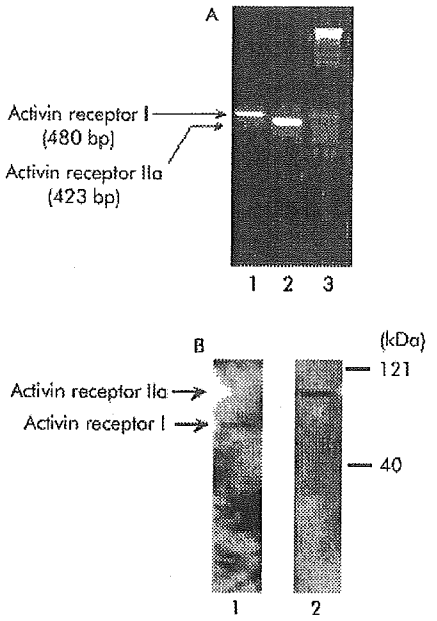

Figure 1 Expression of activin receptors I and IIa in pancreatic stellate cells (PSCs). (A) Reverse transcription-polymerase chain reaction of activin receptors I (lane 1) and IIa (lane 2) was carried out using PSC total RNA (1 μg) as a template. The right lane is a size marker of 1 kb DNA ladder. The bands were confirmed by sequencing. (B) Western blotting of activin receptors I (lane 1) and IIa (lane 2) was performed using their specific antibodies. Molecular markers are indicated on the right.

Activin A is secreted from PSCs

As activin A exerts its effect in an autocrine manner in some cell types,[7 22] we examined whether activin A is secreted from PSCs. As shown in fig 4, activin A peptide was detected in PSC culture medium. In particular, activin A concentration in PSC culture medium markedly increased on the first day after the culture medium was changed to fresh medium. No activin A activity was detected in fresh culture medium. These data indicate that activin A is secreted from PSCs. As an independent test of secretion of activin A from PSCs, we

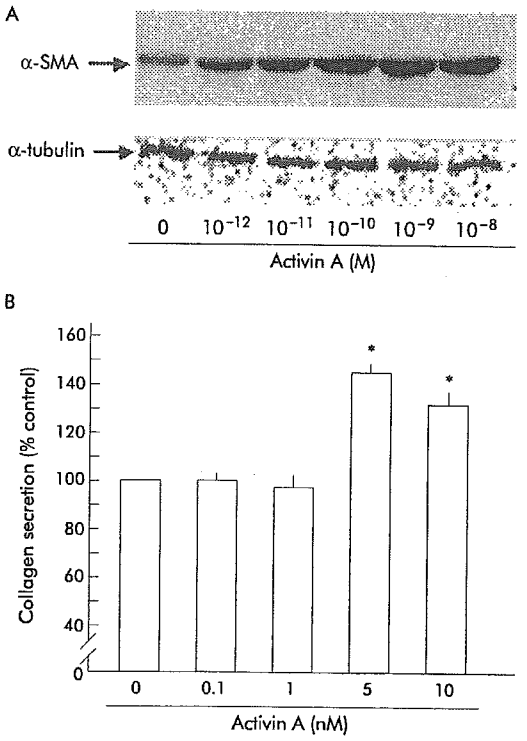

Figure 3 Effect of activin A on α smooth muscle actin (α-SMA) expression and collagen secretion by pancreatic stellate cells (PSCs). (A) Top panel: western blotting of α-SMA in total homogenates of PSCs was carried out after 48 hours of incubation with the indicated amounts of activin A. Activin A increased α-SMA expression of PSCs. Bottom panel: the blots were reprobed with anti-α-tubulin antibody. Expression of α-tubulin protein, examined as an internal control, was not altered by activin A. (B) Collagen secretion by PSCs into culture medium during 48 hours of incubation with the indicated amounts of activin A was quantified with Sirius red dye. Results are expressed as per cent collagen concentration of controls. Values are means (SEM) of three independent experiments (*$p<0.05$).

examined the existence of activin A peptide in PSCs with immunocytochemistry. We applied double staining with an antiactivin A polyclonal antibody and an anti-α-SMA

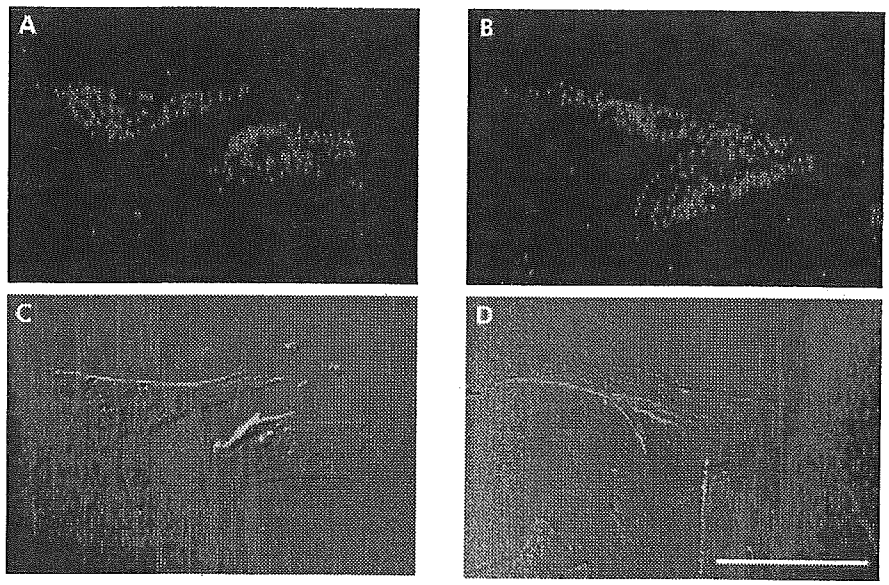

Figure 2 Fluorescence micrographs showing the immunoreactivity of (A) activin receptor type I and (B) activin receptor type IIa in pancreatic stellate cells, with corresponding Nomarski images (C, D). Bar 40 μm.

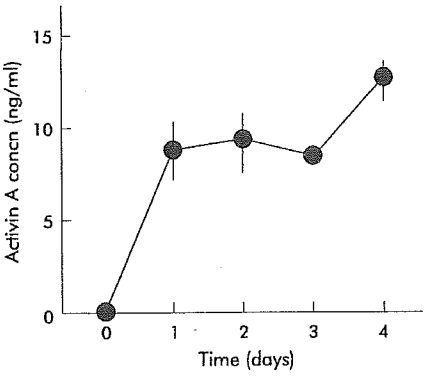

Figure 4 Activin A secretion from pancreatic stellate cells. Activin A concentration in culture medium was determined 1–4 days after the culture medium was changed. Results are means (SEM) of three independent experiments.

monoclonal antibody, a marker of activated PSCs. As depicted in fig 5, activin A signals were observed as small dots in PSCs in which α-SMA signals were detected as fine linear architecture. These data indicate that activin A peptide is present in PSCs, confirming that activin A is secreted from PSCs.

Activin A and TGF-β enhanced each other's secretion and mRNA expression of PSCs

In PSC activation, TGF-β is also known to play a stimulatory role in an autocrine manner.[16] To elucidate the interaction of the stimulatory effects of autocrined activin A and TGF-β on PSC activation, we examined their effects on each other's secretion and mRNA expression of PSCs. As shown in fig 6, activin A enhanced TGF-β secretion from PSCs in a dose dependent manner (fig 6B). In the same manner, TGF-β also increased activin A secretion from PSCs (fig 6A). These data indicate that activin A and TGF-β potentiated each other's secretion from PSCs. Moreover, activin A and TGF-β increased each other's mRNA expression in PSCs in a dose dependent manner (fig 6C, D). These data indicate that activin A and TGF-β coordinately activate PSCs. Moreover, these results provide a hypothesis that blockade of autocrined activin A may be able to decrease TGF-β mRNA expression and TGF-β secretion of PSCs, and consequently reduce PSC activation and collagen secretion by blocking both activin A and TGF-β stimuli.

Follistatin decreased TGF-β secretion and mRNA expression of PSCs

To examine this hypothesis, we investigated the effect of follistatin, an activin A binding protein that blocks activin A activity, on TGF-β secretion and mRNA expression of PSCs. As shown in fig 7, follistatin decreased TGF-β secretion from PSCs in a dose dependent manner (fig 7A). Follistatin also reduced TGF-β mRNA expression in PSCs (fig 7B). Together with the data shown above that activin A enhanced TGF-β secretion from PSCs, it is suggested that follistatin inhibited TGF-β secretion and mRNA expression of PSCs by inhibiting the autocrined activin A stimulus, indicating that follistatin can concurrently inhibit both autocrined activin A and TGF-β stimuli.

Follistatin inhibited PSC activation and collagen secretion

Knowing that follistatin blocks both activin A and TGF-β stimuli to PSCs, we finally examined whether follistatin

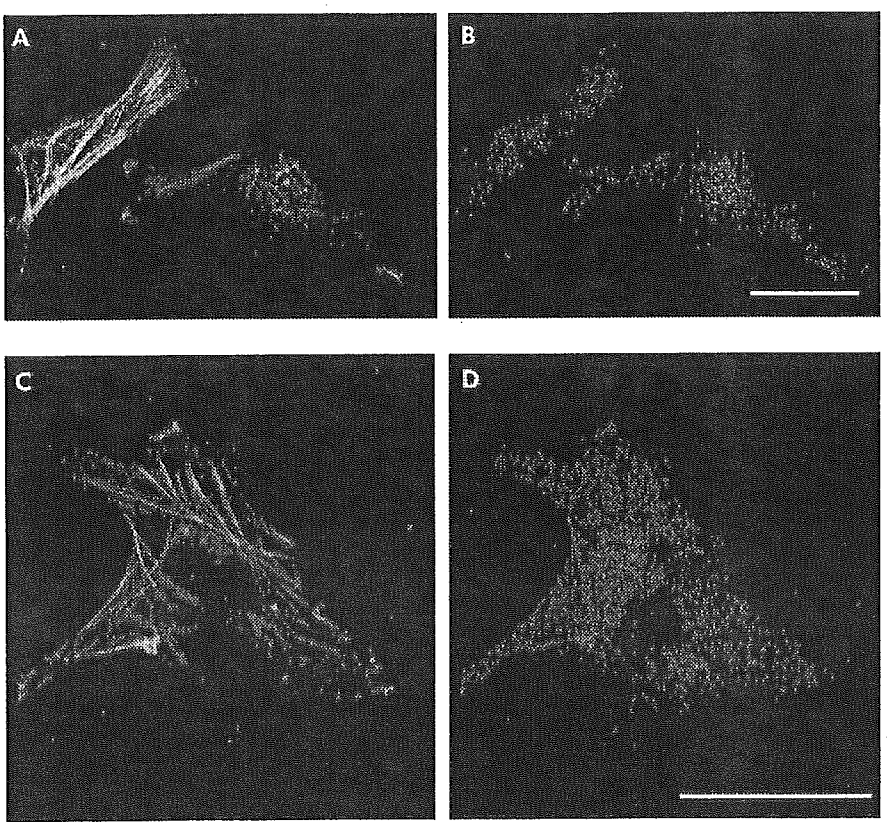

Figure 5 Fluorescence micrographs showing the immunoreactivity of activin A in pancreatic stellate cells (PSCs). Cells were double stained with an anti-activin A rabbit polyclonal antibody (B, D) and an anti-α smooth muscle actin (α-SMA) mouse monoclonal antibody (A, C). Activin A immunoreactivity was observed in PSCs identified with their α-SMA fine network architecture. Bars 40 μm.

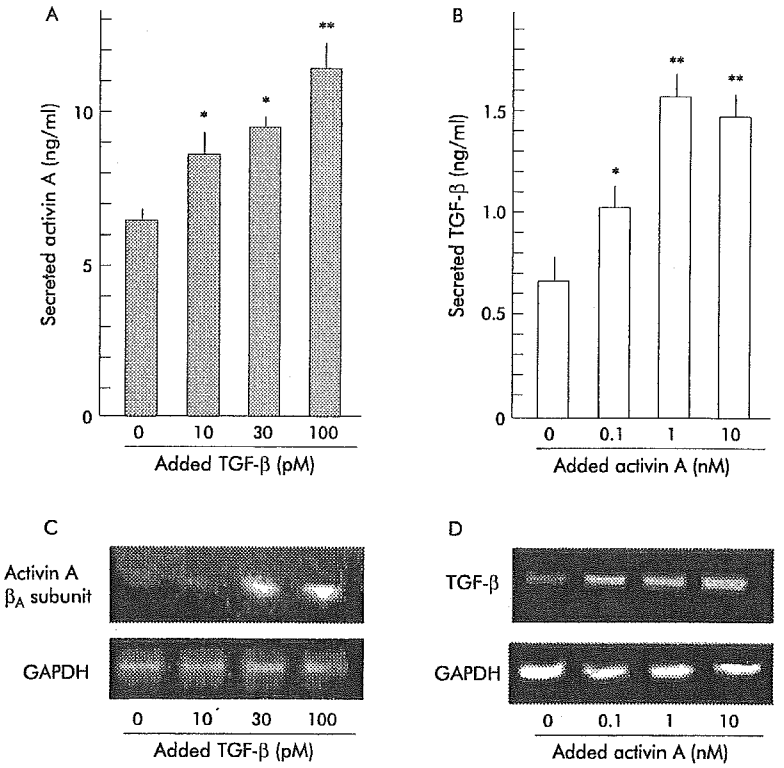

Figure 6 Effect of activin A and transforming growth factor β (TGF-β) on each other's secretion and mRNA expression of pancreatic stellate cells (PSCs). (A, B) Concentration of activin A (A) and TGF-β (B) secreted from PSCs into culture medium was quantified after 48 hours of incubation with the indicated amounts of TGF-β (A) or activin A (B). Results are means (SEM) of three independent experiments (*$p<0.05$, **$p<0.01$). (C, D) mRNA expression of activin A βA subunit (C) and TGF-β (D) was determined by reverse transcription-polymerase chain reaction after 24 hours of incubation with the indicated amounts of TGF-β (C) or activin A (D), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression as a control.

inhibits PSC activation and collagen secretion. As shown in fig 8A, follistatin decreased α-SMA content in PSCs in a dose dependent manner, indicating that follistatin inhibited PSC autoactivation. In addition, as shown in fig 8B, follistatin inhibited collagen secretion by PSCs. These data indicate that follistatin is a potent inhibitor of PSC activation and collagen secretion.

DISCUSSION

In this study, we demonstrated that activin A activates PSCs and promotes collagen secretion in an autocrine manner. We also showed that activin A and TGF-β enhance each other's secretion and mRNA expression of PSCs. We also found that follistatin, an activin A binding and blocking protein, inhibits TGF-β secretion and mRNA expression of PSCs and attenuates PSC activation and collagen secretion. These data suggest the participation of activin A in pancreatic fibrosis and the potential application of follistatin in its treatment.

With regard to the effects of activin A on the pancreas, much attention has been directed towards its physiological role in regulation and differentiation of pancreatic islet cells. Activin A has been shown to be present in progenitor cells of fetal pancreas[23] and in islet A and D cells of adult pancreatic islets.[8] Both in vitro and in vivo studies have revealed that activin A regulates differentiation of pancreatic endocrine cells.[9 18] It is also reported that activin A stimulates insulin release from islet B cells.[8] To date, however, activin A participation in pancreatic disorders, including pancreatic fibrosis, has been uncertain and its presence in PSCs has not been described. Thus the current study provides the first evidence of activin A participation in pancreatic fibrosis.

The mechanism of digestive organ fibrosis has been extensively studied on hepatic fibrosis, including hepatic stellate cell (HSC) participation. Thus the mechanism of pancreatic fibrosis has been assessed in the analogy of that of hepatic fibrosis. Although activin A has been demonstrated to contribute to hepatic fibrosis and to increase HSC collagen expression,[24 25] it is noteworthy that the cellular source of activin A is quite different between the pancreas and liver. In both normal and fibrotic livers, activin A is mainly expressed in parenchymal hepatocytes.[7 24] Although HSCs express activin A,[26] the cellular population of parenchymal hepatocytes is much greater than that of HSCs in the liver. Thus it is assumed that activin A promotes hepatic fibrosis mainly in a paracrine manner. In contrast, pancreatic acinar cells, a major inhabitant of the pancreas, do not express activin A.[8] Although A and D cells in pancreatic islets express activin A, their population is small, especially in fibrotic pancreas. Therefore, it can be postulated that activin A activates PSCs and promotes pancreatic fibrosis in an autocrine manner. Taken together, we suggest that distinct mechanisms underlie pancreatic and hepatic fibrosis.

Our observations that activin A and TGF-β increased each other's mRNA expression and secretion of PSCs are unique. They imply the existence of an autocrine loop of activin A and TGF-β in PSCs. In parenchymal hepatocytes, both activin A and TGF-β are expressed and regulate their proliferation in an autocrine manner.[7 26] However, the interaction between autocrined activin A and TGF-β in hepatocyte growth control is still unknown. The current study demonstrated, for the first time, the interaction between autocrined activin A and TGF-β, suggesting activin A and TGF-β synergistically promote pancreatic fibrosis. Although this interaction implies that both activin A and TGF-β are potent fibrogenic factors, it provides a novel therapeutic strategy for pancreatic fibrosis. Accordingly, blockade of either activin A or TGF-β can inhibit

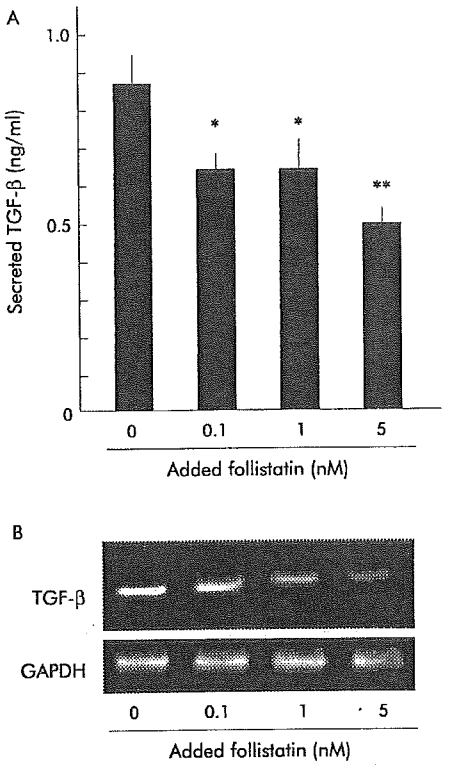

Figure 7 Effect of follistatin on transforming growth factor β (TGF-β) secretion and mRNA expression of pancreatic stellate cells (PSCs). (A) Concentration of TGF-β secreted from PSCs into culture medium was quantified after 48 hours of incubation with the indicated amounts of follistatin. Results are means (SEM) of three independent experiments (*p<0.05, **p<0.01). (B) mRNA expression of TGF-β in PSCs was determined by reverse transcription-polymerase chain reaction after 24 hours of incubation with the indicated amounts of follistatin, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA

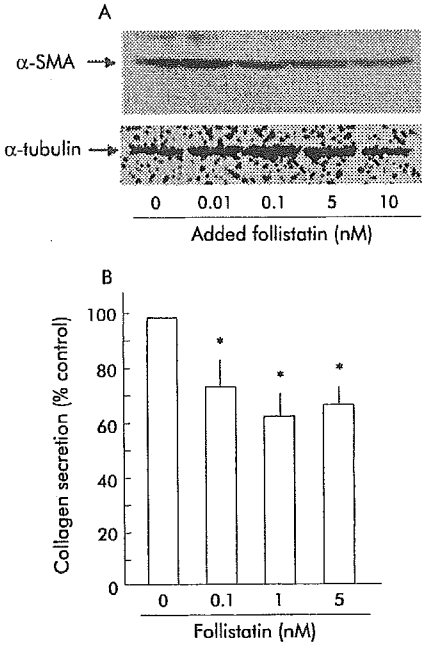

Figure 8 Effect of follistatin on pancreatic stellate cell (PSC) activation and collagen secretion. (A) Top panel: western blotting of α smooth muscle actin (α-SMA) in total homogenates of PSCs was carried out after 48 hours of incubation with the indicated amounts of follistatin. Follistatin reduced α-SMA expression of PSCs. Bottom panel: the blots were reprobed with anti-α-tubulin antibody. Expression of α-tubulin protein, examined as an internal control, was not altered by activin A. (B) Collagen secretion by PSCs into culture medium during 48 hours of incubation with the indicated amounts of follistatin was quantified with Sirius red dye. Results are expressed as per cent collagen concentration of control. Values are means (SEM) of three independent experiments (*p<0.05).

both effects of activin A and TGF-β on PSC activation. Indeed, follistatin reduced TGF-β secretion and mRNA expression of PSCs and potently inhibited PSC activation and collagen secretion (figs 7, 8). These results suggest that follistatin may be a potential therapeutic agent to inhibit pancreatic fibrosis. In this respect, it has been reported that both intraportally and intravenously administrated follistatin accelerated liver regeneration after partial hepatectomy,[27 28] suggesting follistatin can play a therapeutic role in vivo. Thus an in vivo study of the inhibitory effects of follistatin on pancreatic fibrosis is warranted.

Although follistatin is a potent inhibitor of TGF-β expression and collagen secretion of PSCs, even high doses of follistatin could not cause complete loss of TGF-β expression or of collagen secretion (figs 7, 8). Recently, Shek *et al* reported that blockade of autocrined TGF-β by its neutralising antibodies could not completely abolish collagen synthesis by PSCs, which is consistent with our observations.[29] These data suggest the involvement of additional mechanisms in the regulation of TGF-β and collagen expression in PSCs apart from the autocrine loop of activin A and TGF-β. In this respect, Mews *et al* reported that collagen synthesis of PSCs is enhanced by various cytokines such as tumour necrosis factor α and interleukin 10.[30] In other cell types, TGF-β expression is increased by interleukin 1[31] and platelet derived growth factor,[32] to which PSCs respond.[30] Although it is still uncertain that these cytokines are secreted from PSCs, HSCs have been shown to express and secrete a variety of cytokines, including interleukins.[33–35] Thus the possibility that autocrined cytokines other than activin A and TGF-β might regulate collagen and TGF-β expression in PSCs deserves further investigation.

In addition to the effect on cellular differentiation and activation, activin A possesses inhibitory effect on the proliferation of various cells, including pancreatic acinar cells.[36] However, exogenous activin A (1–10 nM) did not alter PSC ^{3}H-thymidine uptake. In addition, follistatin (1–10 nM) increased it by only 10–15% (our unpublished data). Thus the inhibitory effect of activin A on PSC proliferation may be, if any, much smaller than that of TGF-β.[16]

In conclusion, we have shown that activin A is an autocrine activator of PSCs and follistatin inhibited PSC activation. These observations provide new insights for understanding the mechanism of pancreatic fibrosis and developing novel therapeutic strategies.

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Distinct Roles of Smad2-, Smad3-, and ERK-dependent Pathways in Transforming Growth Factor-β_1 Regulation of Pancreatic Stellate Cellular Functions*

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Pancreatic stellate cells (PSCs) play a major role in promoting pancreatic fibrosis. Transforming growth factor-β_1 (TGF-β_1) regulates PSC activation and proliferation in an autocrine manner. The intracellular signaling pathways of the regulation were examined in this study. Immunoprecipitation and immunocytochemistry revealed that Smad2, Smad3, and Smad4 were functionally expressed in PSCs. Adenovirus-mediated expression of Smad2, Smad3, or dominant-negative Smad2/3 did not alter TGF-β_1 mRNA expression level or the amount of autocrine TGF-β_1 peptide. However, expression of dominant-negative Smad2/3 inhibited PSC activation and enhanced their proliferation. Co-expression of Smad2 with dominant-negative Smad2/3 restored PSC activation inhibited by dominant-negative Smad2/3 expression without changing their proliferation. By contrast, co-expression of Smad3 with dominant-negative Smad2/3 attenuated PSC proliferation enhanced by dominant-negative Smad2/3 expression without altering their activation. Exogenous TGF-β_1 increased TGFβ_1 mRNA expression in PSCs. However, PD98059, a specific inhibitor of mitogen-activated protein kinase kinase (MEK1), inhibited ERK activation by TGF-β_1, and consequently attenuated TGF-β_1 enhancement of its own mRNA expression in PSCs. We propose that TGF-β_1 differentially regulates PSC activation, proliferation, and TGF-β_1 mRNA expression through Smad2-, Smad3-, and ERK-dependent pathways, respectively.

Pancreatic stellate cells (PSCs)[1] were recently identified, isolated, and characterized (1, 2). In the normal pancreas, PSCs possess fat droplets containing vitamin A and are quiescent. In the quiescent state they are characterized by desmin-positive but α-smooth muscle actin (α-SMA)-negative staining (1). When cultured *in vitro*, PSCs are autoactivated (autotransformed), changing their morphological and functional features (2). PSCs commence losing vitamin A containing lipid droplets, highly proliferating, increasing expression of α-SMA, and producing and secreting extracellular matrix components such as collagen and fibronectin. Namely, PSCs are autotransformed to myofibroblast-like cells. *In vivo*, PSCs are also activated during both human and experimental pancreatic fibrosis (3). Therefore, PSCs are believed to play an important role in pancreatic fibrogenesis.

TGF-β_1 is one of major profibrogenic cytokines in various tissues. Recently, evidence for TGF-β participation in pancreatic fibrogenesis has been mounting. In this regard, it has been noted that transgenic mice overexpressing TGF-β_1 in islet cells develop fibrosis of exocrine pancreas (4). Moreover, inhibition of TGF-β_1 by anti-TGF-β_1 antibody reduced extracellular matrix production in rat cerulein pancreatitis (5). TGF-β_1 has also been shown to promote PSC activation and collagen production and to inhibit proliferation of PSCs in an autocrine manner (6, 7). In human chronic pancreatitis tissue, TGF-β_1 expression was observed in acinar cells adjacent to areas of fibrosis and in spindle cells in fibrotic bands (3). Thus, TGF-β_1 is thought to promote pancreatic fibrosis, in part by modulating PSC functions. However, the intracellular signaling pathway(s) through which TGF-β_1 regulates PSC functions is still uncertain.

Sma- and Mad-related proteins (Smads) are a group of recently identified molecules that function as intracellular signaling mediators and modulators of TGF-β family members (8, 9). Smads can be classified into three groups: receptor-regulated Smads (R-Smads), common mediator Smad (Co-Smads), and inhibitory Smads (I-Smad). In TGF-β signaling pathway, Smad2 and Smad3 function as R-Smads, Smad4 functions as a Co-Smad, and Smad7 functions as an I-Smad. Upon TGF-β binding to TGF-β type II receptor, the type II receptor kinase phosphorylates the GS domain of TGF-β type I receptor, leading to activation of the type I receptor. The activated type I receptor kinase phosphorylates Smad2 and Smad3 (R-Smads) at two serine residues in the SSXS motif at their extreme C termini (10, 11). Phosphorylated Smad2 and Smad3 form oligomeric complexes with Smad4 (Co-Smad); the complexes then translocate into the nucleus. These complexes then activate the transcription of target genes. Thus, TGF-β intracellular signaling involves dual Smad-dependent pathways, namely, Smad2-

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[1] The abbreviations used are: PSC, pancreatic stellate cell; α-SMA, α-smooth muscle actin; Smad, Sma- and Mad-related protein; R-Smad, receptor-regulated Smad; Co-Smad, common mediator Smad; I-Smad, inhibitory Smad; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; TGF, transforming growth factor; pfu, plaque-forming units; HSC, hepatic stellate cell(s).

and Smad3-dependent pathways. In addition to Smad-mediated signaling pathways, other signaling pathways have also been shown to mediate TGF-β signaling. For example, TGF-β activates Rho-GTPase, mitogen-activated protein kinases, and protein kinase B (12). However, a direct link between these mediators and TGF-β receptors has not been demonstrated unequivocally (12).

Because TGF-β_1 stimulus is transduced through the multiple intracellular pathways described above, the elucidation of the signaling pathways through which TGF-β_1 regulates PSC functions is likely to provide new insights related to the molecular pathogenesis of pancreatic fibrosis. We therefore conducted the present study to examine these pathways by applying adenovirus-mediated overexpression of Smad2 and Smad3. However, because Smad2 and Smad3 compete with each other at the receptor and for Smad4-binding steps for their activation, overexpression of Smad2 and Smad3 blocks endogenous Smad3 and Smad2 functions by competing at the binding steps to TGF-β receptor and Smad4. Thus, the possibility remains that the observed effects of Smad2 and Smad3 overexpression on PSC functions may result from the blockade of endogenous Smad3 and Smad2 function but may not result from the enhancement of Smad2 and Smad3 activity by their overexpression. To exclude this possibility, we investigated Smad2- and Smad3-specific roles in TGF-β_1 regulation of PSC functions by co-expression of dominant-negative Smad2/3 with Smad2 or Smad3. Although the dominant-negative Smad2/3 mutant was generated by substituting Glu for Asp-407 of Smad3, which is defective in TGF-β receptor-dependent phosphorylation, this mutant possesses a dominant-negative effect on both Smad2 and Smad3 (13). Thus, we designated the mutant as dominant-negative Smad2/3. In this way, expression of dominant-negative Smad2/3 blocks both endogenous Smad2 and Smad3 functions at TGF-β receptor-dependent phosphorylation step. Therefore, the co-expression of Smad2 or Smad3 with dominant-negative Smad2/3 rescues only Smad2- or Smad3-dependent pathways, respectively. Thus, we can examine Smad2- and Smad3-specific signaling pathways. Using this method, we demonstrated that TGF-β_1 activates PSCs through a Smad2-dependent pathway and inhibits their proliferation through a Smad3-dependent pathway. Moreover, TGF-β_1 enhanced its own mRNA expression and peptide secretion of PSCs through an ERK-dependent pathway.

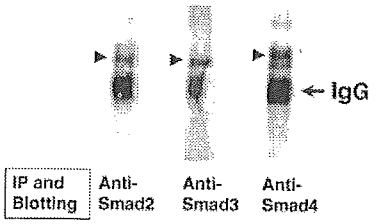

FIG. 1. **Immunoprecipitation of Smad2, Smad3, and Smad4.** Smad proteins were immunoprecipitated (*IP*) from PSC lysate using polyclonal antibodies against each Smad protein. The immunoprecipitates were fractioned on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Western blotting of the immunoprecipitates was carried out with the same antibodies used for each immunoprecipitation.

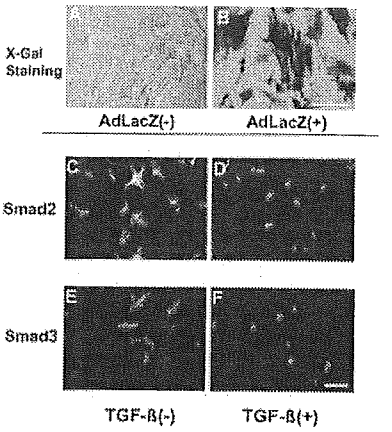

FIG. 2. **TGF-$\beta_1$induces nuclear translocation of both Smad2 and Smad3 in PSCs.** Immunocytochemical analysis of nuclear accumulation of Smad2 and Smad3 was performed using PSCs that were transfected with Smad2 or Smad3 adenoviral vectors and overexpressed the proteins. *A* and *B*, infection efficiency of adenovirus was determined by using AdLacZ infection and *in situ* staining with X-gal. *C–F*, PSCs were infected with AdSmad2 (*C* and *D*) or AdSmad3 (*E* and *F*). The cells were stained with anti-Smad2 (*C* and *D*) or anti-Smad3 (*E* and *F*) antibodies before (*C* and *E*) and after (*D* and *F*) 2 h of stimulation with 10 pM TGF-β_1. *Bars*, 40 μm.

EXPERIMENTAL PROCEDURES

Materials—Nycodenz, Pronase, and anti-α-SMA antibody were purchased from Sigma. DNase I and collagenase P were from Roche Applied Science. Anti-Smad2, anti-Smad3, anti-Smad4, and anti-ERK antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-phosphorylated ERK was from Cell Signaling (Beverly, MA). Horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG, HRP-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG, and Cy3-conjugated donkey anti-goat IgG antibodies were from Jackson ImmunoResearch (West Grove, PA). PD98058 was from Calbiochem (San Diego, CA).

Isolation and Culture of Rat Pancreatic Stellate Cells—Rat pancreatic stellate cells were prepared as described (1). Briefly, rat pancreas was digested in Gey's balanced salt solution supplemented with 0.05% collagenase P, 0.02% Pronase, and 0.1% DNase. After filtration through nylon mesh, the cells were centrifuged on a 13.2% Nycodenz gradient at 1400 $\times$ *g* for 20 min. PSCs were collected from the band just above the interface of the Nycodenz solution and the aqueous layer, washed, and resuspended in Iscove's modified Dulbecco's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. PSCs were cultured in a 5% CO_2 atmosphere at 37 °C. All of the experiments were carried out using PSCs from passages 2 and 3.

Immunoprecipitation and Western Blotting—Immunoprecipitation was performed as described previously (14). Western blotting was carried out as described before (15), using enhanced chemiluminescence reagent to visualize the secondary antibody.

Adenovirus Infection—Recombinant adenoviruses containing recombinant Smad DNAs were kindly provided by Dr. Miyazono (University of Tokyo, Tokyo, Japan). For a single adenovirus infection, the cells were infected with a recombinant adenovirus at a dose of 10 plaque-forming units (pfu)/cell in the culture media described above. In the experiments using double adenovirus infection, the cells were infected with dominant-negative Smad2/3 adenovirus (AdDNSmad2/3) at a dose of 10 pfu/cell, simultaneously with Smad2 (AdSmad2) or Smad3 (AdSmad3) adenovirus at doses of 1, 5, or 10 pfu/cell. Subsequent experiments were performed 48 h after infection. An adenovirus expressing β-galactosidase (AdLacZ) was used as an infection control.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (15, 16). The samples were examined by epifluorescence microscopy (see Fig. 2) and confocal fluorescence microscopy (see Fig. 3) (Fluoview FV300; Olympus, Tokyo, Japan) using an Olympus BX51 microscope. The images were digitized and then processed using Photoshop 5.0 software (Adobe Systems Inc., Mountain View, CA).

Measurement of DNA Synthesis—DNA synthesis was determined by measuring [^{3}H]thymidine incorporation into cells. [^{3}H]Thymidine was added to the culture medium and incubated for 2 h, and the incorporation of radioactivity was measured as described previously (17).

Measurement of TGF-β_1 Peptide Secretion—TGF-β_1 peptide secretion was examined by determining the concentration of TGF-β_1 peptide in a culture medium of PSCs using a commercial kit from DRG International (Mountainside, NJ), according to the manufacturer's instructions.

Competitive Reverse Transcription-PCR of TGF-β_1 mRNA—Total RNA was obtained from PCS cells by using ISOGEN (Wako, Tokyo, Japan), followed with synthesis of double-stranded DNA as described

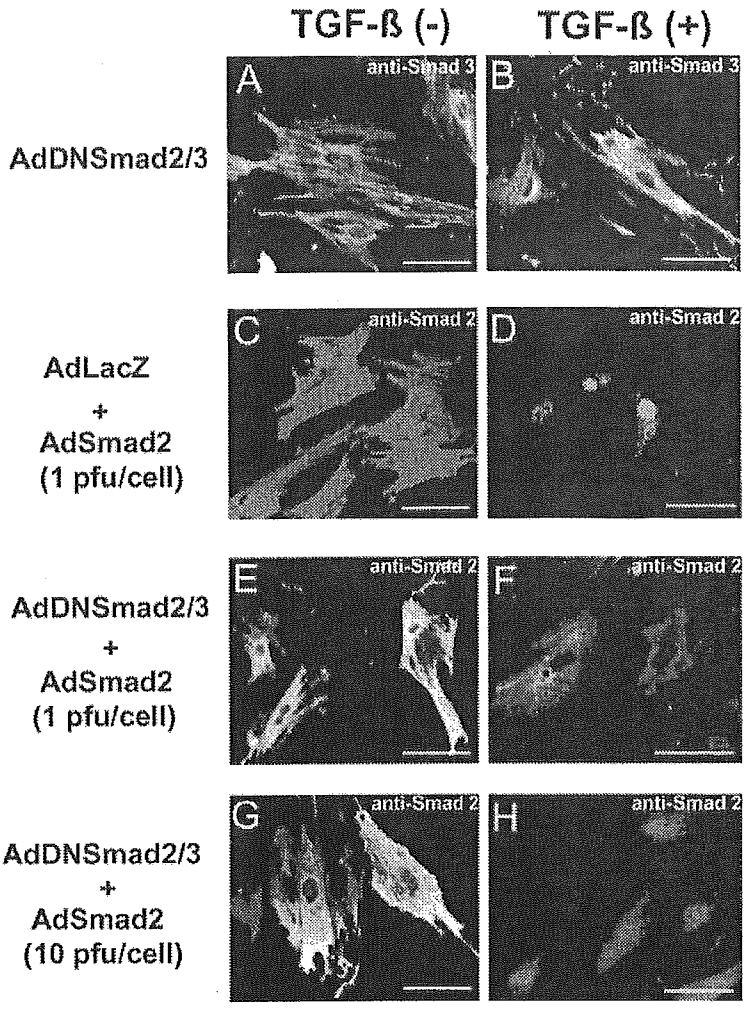

FIG. 3. **Characterization of dominant-negative Smad2/3 expressed in PSCs.** PSCs were infected with 10 pfu/cell AdDNSmad2/3 (*A* and *B*) or combinations of 10 pfu/cell AdLacZ + 1 pfu/cell AdSmad2 (*C* and *D*), 10 pfu/cell AdDNSmad2/3 + 1pfu/cell AdSmad2 (*E* and *F*), or 10 pfu/cell AdDNSmad2/3 + 10 pfu/cell AdSmad2 (*G* and *H*). The cells were stained with anti-Smad2 (*C–H*) or anti-Smad3 (*A* and *B*) antibodies before (*A*, *C*, *E*, and *G*) and after (*B*, *D*, *F*, and *H*) 2 h of stimulation with 10 pM TGF-β. *Bars*, 40 μm.

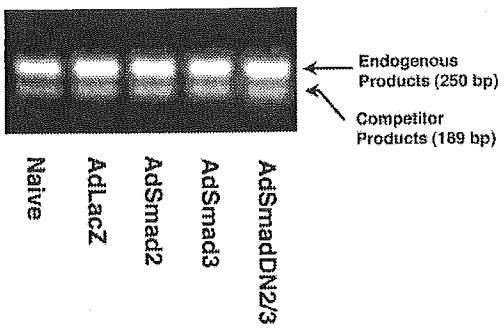

FIG. 4. **Overexpression of Smad2, Smad3, or dominant-negative Smad2/3 did not alter TGF-β_1 mRNA expression level in PSCs.** PSCs were infected with AdLacZ, AdSmad2, AdSmad3, or AdDNSmad2/3. TGF-β_1 mRNA expression in PSCs was determined with competitive reverse transcription-PCR 48 h after the infection.

previously (18). Competitive PCR of TGF-β_1 mRNA was performed using the competitive PCR kit for rat TGF-β_1 (Maxim Biotech Inc., San Francisco, CA) according to the manufacturer's instructions. In this method, 189- and 250-bp PCR fragments are generated by amplifying a DNA competitor and rat TGF-β_1 cDNA, respectively.

RESULTS

Expression of Smad2, Smad3, and Smad4 Proteins in PSCs—We first examined the expression of R-Smads (Smad2 and Smad3) and Co-Smad (Smad4) in rat PSCs. To this end, we performed immunoprecipitation of Smad2, Smad3, and Smad4 from crude extract of rat PSCs, using antibodies specific to each Smad. As shown in Fig. 1, all of the three Smads were immunoprecipitated from rat PSCs, suggesting that essential components of both Smad2- and Smad3-dependent TGF-β_1 signaling pathways are present in PSCs.

TABLE I
TGF-β_1 peptide concentration (ng/ml) in the culture medium of PSCs infected with AdSmad2, AdSmad3, or AdDNSmad2/3

TGF-β_1 peptide concentration was determined in PSC culture medium 2 days after each adenovirus infection using enzyme-linked immunosorbent assay. The values are expressed as the means ± S.E. for three independent experiments. DN, dominant-negative.

LacZ	2.65 ± 0.45
Smad2	2.23 ± 0.22
Smad3	2.55 ± 0.28
DN-Smad2/3	2.26 ± 0.36

TGF-β Induced the Nuclear Accumulation of both Smad2 and Smad3 in PSCs—We next examined whether Smad2- and Smad3-dependent TGF-β_1 signaling pathways are functioning in PSCs. Because the levels of endogenously expressed Smad2 and Smad3 are not sufficient to detect by immunochemistry using specific antibodies, we used adenovirus-mediated overexpression of Smad2 and Smad3 in PSCs. We first determined the infection efficiency by using AdLacZ infection and *in situ* staining with X-gal. As shown in Fig. 2, more than 98% of

AdLacZ-infected PSCs expressed β-galactosidase (Fig. 2, *A* and *B*). In PSCs infected with AdSmad2 and AdSmad3, sufficient expression of Smad2 and Smad3 was observed by immunocytochemistry (Fig. 2, *C* and *E*). When treated with 10 pM TGF-β_1, both Smad2 and Smad3 accumulated in the nucleus (Fig. 2, *D* and *F*). These data indicate that functional Smad2- and Smad3-dependent signaling pathways are present in PSCs.

Infection Efficacy and Characterization of AdDNSmad2/3 in PSCs—We subsequently attempted to elucidate the intracellular signaling pathways through which autocrine TGF-β_1 modulates PSC functions using AdSmad2, AdSmad3, and AdDNSmad2/3. As described above, infection of AdSmad2 and AdSmad3 into cells resulted in expression of sufficient quantities of functional Smad2 and Smad3 proteins in PSCs, respectively. Thus, we next characterized AdDNSmad2/3 infection in PSCs with confocal immunofluorescence microscopy. In this experiment, we utilized anti-Smad3 antibody to identify dominant-negative Smad2/3 protein expression because AdDNSmad2/3 was generated by substituting Glu for Asp-407 of smad3. As shown in Fig. 3, AdDNSmad2/3 infection induced expression of sufficient quantities of dominant-negative Smad2/3 protein throughout the cytoplasm in PSCs (Fig. 3*A*). When stimulated with 10 pM TGF-β_1, dominant-negative Smad2/3 did not accumulate in the nucleus but remained in the cytoplasm (Fig. 3*B*). These data demonstrate that the expressed dominant-negative Smad2/3 protein is functionally defective in TGF-β signaling. In COS7 cells, the expression of dominant-negative Smad2/3 has been shown to inhibit both Smad2- and Smad3-dependent TGF-β signaling pathways by blocking Smad2 and Smad3 phosphorylation by TGF-β type 1 receptor (13). We thus performed the next experiments to confirm the dominant-negative nature of AdDNSmad2/3 infection in TGF-β signaling in PSCs. To this end, we investigated the effect of AdDNSamd2/3 and AdSmad2 double infection on Smad2 function in PSCs. When AdLacZ (10 pfu/cell) and AdSmad2 (1 pfu/cell) were co-infected, overexpressed Smad2 accumulated in the nucleus after stimulation with 10 pM TGF-β_1 (Fig. 3, *C* and *D*). In contrast, when AdDNSmad2/3 (10 pfu/cell) was co-infected with AdSmad2 (1 pfu/cell) into PSCs, overexpressed Smad2 did not accumulate in the nucleus but remained in the cytoplasm (Fig. 3, *E* and *F*). These data imply that dominant-negative Smad2/3 blocks Smad2 nuclear accumulation after TGF-β stimulation, indicating the dominant-negative effect of AdSmad2/3 infection on Smad-dependent TGF-β signaling pathway in PSCs. We further examined the effect of higher dose infection of AdSmad2 on the dominant-negative effect of AdSmad2/3 infection. When AdSmad2 was infected at the dose of 10 pfu/cell with AdDNSmad2/3 (10 pfu/cell), overexpressed Smad2 accumulated in the nucleus after stimulation with 10 pM TGF-β_1, although it remained in the cytoplasm to some extent (Fig. 3, *G* and *H*). These data indicate that the Smad2- and Smad3-dependent signaling pathways attenuated by AdDNSmad2/3 infection can be rescued by higher doses of co-infected AdSmad2 or AdSmad3.

Infection of AdSmad2, AdSmad3, or AdDNSmad2/3 Did Not Alter TGF-β_1 mRNA Expression or TGF-β_1 Peptide Secretion of PSCs—After the evaluation of the effects of infection of these adenoviruses on TGF-β signaling pathways in PSCs as described above, we examined whether their infection into PSCs alters TGF-β_1 mRNA expression level and the amount of autocrine TGF-β_1 peptide of PSCs. As shown in Fig. 4, TGF-β_1 mRNA expression level of PSCs was not altered by the infection of AdSmad2, AdSmad3, or AdDNSmad2/3. In addition, TGF-β_1 peptide secretion from PSCs was not affected by their infection (Table I). These data indicate that we can observe the effect of

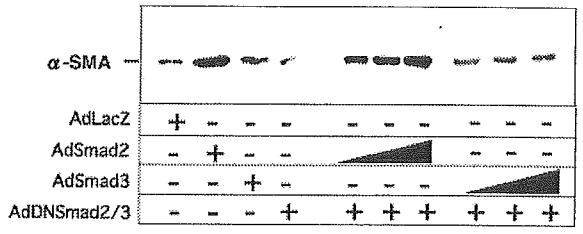

FIG. 5. **Effects of Smad2, Smad3, and dominant-negative Smad2/3 overexpression on α-SMA expression in PSCs.** PSCs were infected with AdLacZ, AdSmad2, AdSmad3, or AdDNSmad2/3. For double adenovirus infection experiments, PSCs were infected with the combination of AdSmad2 and AdDNSmad2/3 or that of AdSmad3 and AdDNSmad2/3. After 48 h of incubation, total homogenates were prepared from PSCs, and aliquots of 10 μg of protein of the homogenates were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was carried out using anti-α-SMA monoclonal antibodies. The results shown are representative of three independent experiments.

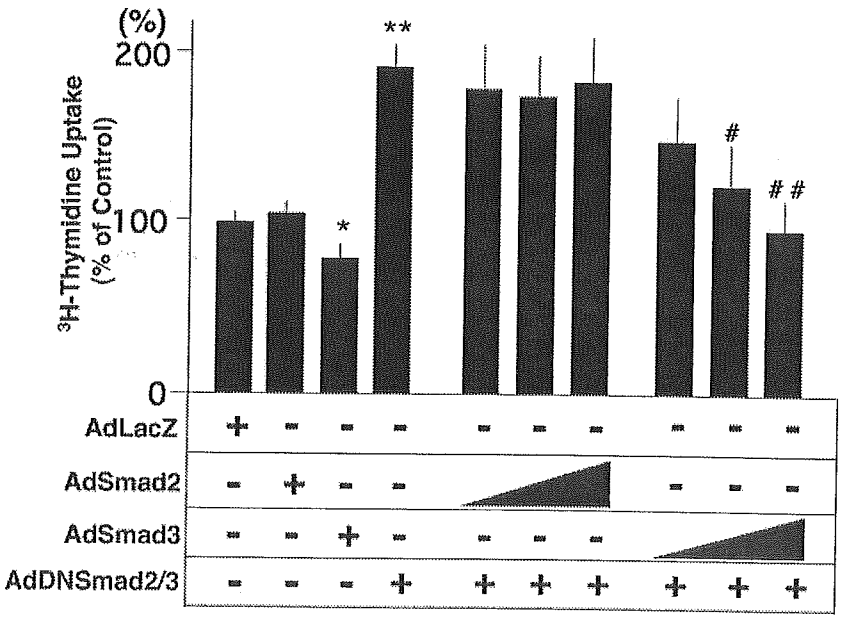

FIG. 6. **Effects of Smad2, Smad3, and dominant-negative Smad2/3 overexpression on DNA synthesis in PSCs.** PSCs were infected with AdLacZ, AdSmad2, AdSmad3, or AdDNSmad2/3. For double adenovirus infection experiments, PSCs were infected with the combination of AdSmad2 and AdDNSmad2/3 or that of AdSmad3 and AdDNSmad2/3. After 48 h of incubation, DNA synthesis was determined with a [^{3}H]thymidine uptake assay. The values are expressed as the means ± S.E. for three independent experiments. *, $p < 0.05$; **, $p < 0.01$, by analysis of variance.

these adenoviruses infections on diverse PSC functions modulated by autocrine TGF-β_1, regardless of the effect of the infection on the amount of autocrine TGF-β_1.

Effects of Smad2, Smad3, and Dominant-negative Smad2/3 Overexpression on PSC Activation—We examined involvement of Smad2 and Smad3 in PSC activation using adenovirus-mediated overexpression of the proteins. PSC activation was examined by determining the amount of α-SMA protein in PSCs with Western blotting. Overexpression of dominant-negative Smad2/3 inhibited PSC activation (Fig. 5), indicating that TGF-β_1 activates PSCs through a Smad-dependent pathway. Moreover, overexpression of Smad2 but not Smad3 enhanced PSC activation, suggesting that TGF-β_1 activates PSCs through a Smad2-dependent but not a Smad3-dependent pathway. However, because both Smad2 and Smad3 compete with each other for receptor and for Smad4 binding steps for the activation of their pathways as described above, the possibility remained that the positive effect of Smad2 overexpression on PSC activation may have resulted from inhibition of endogenous Smad3 function and not the involvement of Smad2-dependent pathway in TGF-β_1 induced PSC activation. To exclude this possibility, we investigated Smad2- and Smad3-specific roles in TGF-β_1-induced PSC activation by co-infection of AdDNSmad2/3 with AdSmad2 or AdSmad3. As shown in Fig. 5, AdSmad2 co-infection with AdDNSmad2/3 rescued PSC activation inhibited by AdDNSmad2/3. On the other hand, AdSmad3 co-infection with AdDNSmad2/3 did not alter PSC activation inhibited by AdDNSmad2/3. These data suggest that TGF-β_1 activates PSCs through a Smad2-dependent pathway .

Effects of Smad2, Smad3, and Dominant-negative Smad2/3 Overexpression on PSC Proliferation—We next examined the pathway through which TGF-β_1 inhibits PSC proliferation. PSC proliferation was examined by determining DNA synthesis by means of [^{3}H]thymidine incorporation. AdDNSmad2/3 infection enhanced PSC proliferation, whereas AdSmad3 infection inhibited it (Fig. 6), suggesting that TGF-β_1 inhibits PSC proliferation through a Smad3-dependent pathway. Moreover, results of the co-infection method showed that AdSmad3 co-infection with AdDNSmad2/3 inhibited PSC proliferation enhanced by AdDNSmad2/3. On the other hand, AdSmad2 co-infection with AdDNSmad2/3 did not alter PSC proliferation augmented by AdDNSmad2/3 infection. These data suggest that TGF-β_1 inhibits PSC proliferation through a Smad3-dependent pathway.

TGF-β_1 Enhanced TGF-β_1 mRNA Expression of PSCs through an ERK-dependent Pathway—As described above, TGF-β_1 mRNA expression of PSCs was not affected by the infection of AdSmad2, AdSmad3, or AdDNSmad2/3, suggesting that the regulation of TGF-β_1 mRNA expression in PSCs is independent of Smad-dependent signaling pathways. Thus, we next attempted to elucidate the regulatory mechanism of TGF-β_1 mRNA expression in PSCs. To this end, we first examined whether TGF-β_1 modulates its own mRNA expression in PSCs. As shown in Fig. 7*A*, the addition of exogenous TGF-β_1 into the culture medium of PSCs enhanced TGF-β_1 mRNA expression in a dose-dependent manner, indicating TGF-β_1 autoinduction independent of Smad-mediated signaling. Because mitogen-activated protein kinases including ERKs are also TGF-β signaling mediators (12), we examined the participation of ERK-dependent pathway in the autoinduction of TGF-β_1 mRNA in PSCs. For this purpose, we blocked ERK activation by using the MEK1 inhibitor PD98059. PD98059 pretreatment decreased TGF-β_1 mRNA expression in PSCs (Fig. 7*B*, *first* and *second lanes*). Moreover, the addition of exogenous TGF-β_1 into the culture medium could not enhance TGF-β_1 mRNA expression in PSCs pretreated with PD98059

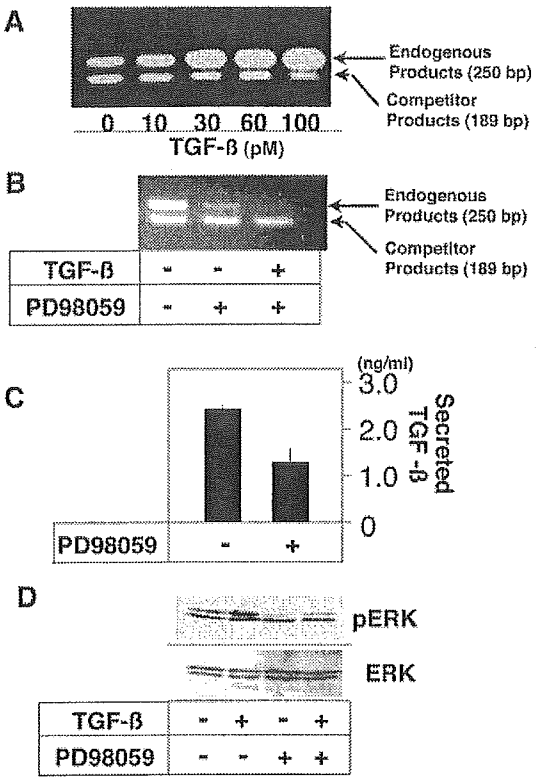

FIG. 7. **Effect of MEK1 inhibitor PD98059 on TGF-β_1 autoinduction in PSCs.** *A*, cultured PSCs were incubated for 48 h with the indicated amounts of TGF-β_1. After incubation, TGF-β_1 mRNA expression in PSCs was determined by competitive reverse transcription-PCR. *B*, After 2 h of pretreatment with (*second* and *third lanes*) or without (*first lane*) 10 nM PD98059, cultured PSCs were incubated for 24 h in the presence (*third lane*) or absence (*first* and *second lanes*) of 100 pM TGF-β_1. *C*, TGF-β_1 peptide secreted into culture medium during 48 h of incubation in the presence (*right column*) or absence (*left column*) of 10 nM PD 98059 was determined with enzyme-linked immunosorbent assay. *D*, After 2 h of pretreatment with (*third* and *fourth lanes*) or without (*first* and *second lanes*) 10 nM PD98059, cultured PSCs were incubated for 30 min in the presence (*second* and *fourth lanes*) or absence (*first* and *third lanes*) of 100 pM TGF-β_1. The activation of ERK was then determined by Western blotting using anti-phosphorylated ERK1/2 antibody (*upper panel*). Western blotting with anti-ERK1/2 antibody (*lower panel*) was carried out as an internal control.

(Fig. 7*B*, *first* and *second lanes*). Consistent with these data, PD98059 pretreatment decreased TGF-β_1 peptide secretion from PSCs (Fig. 7*C*). Finally, we confirmed that TGF-β_1 activates ERK in PSCs (Fig. 7*D*, *first* and *second lanes*), and PD98059 pretreatment blocked ERK activation (Fig. 7*D*, *third* and *fourth lanes*). These data indicate that TGF-β_1 autoinduction in PSCs is regulated through an ERK-dependent pathway.

DISCUSSION

In this study, we demonstrated that TGF-β_1 regulates various PSC functions through distinct intracellular signaling pathways. Adenovirus-mediated dominant-negative Smad2/3 expression inhibited PSC activation and enhanced their proliferation but did not alter TGF-β_1 mRNA expression. Co-expression of Smad2 with dominant-negative Smad2/3 restored the PSC activation inhibited by dominant-negative Smad2/3 expression. In contrast, co-expression of Smad3 with dominant-negative Smad2/3 attenuated the proliferation enhanced by dominant-negative Smad2/3 expression. Moreover, exogenous TGF-β_1 increased TGF-β_1 mRNA expression in PSCs, and MEK1 inhibitor PD98059 blocked it. Accordingly, TGF-β_1 enhances PSC activation, inhibits their proliferation, and in-

creases their TGF-β_1 mRNA expression through Smad2-, Smad3-, and ERK-dependent pathways, respectively.

Although TGF-β signaling is mediated by both Smad2- and Smad3-dependent pathways, their functional difference has been uncertain. However, studies using targeted homozygous deletion of Smad2 and Smad3 genes in mice revealed their distinct functions in embryo development. Smad2 knockout mice are embryonic lethal because of the defects of left-right patterning and mesoderm induction (19, 20). In contrast, Smad3 knockout mice are viable but are smaller than wild-type littermates and show forelimb malformation and die because of immune function defects (21, 22). In addition, using hepatic stellate cells (HSCs) derived from the Smad3 knockout mice, Schnabl *et al.* (23) recently elucidated the Smad3 specific role in cellular function. They reported that Smad3 is necessary for TGF-β-mediated inhibition of HSC proliferation but not for HSC activation, which is consistent with our data on Smad3 function in PSC proliferation. However, the specific role of Smad2 in HSC function has not yet been demonstrated. Recently, using fibroblasts derived from both embryos of Smad2 and Smad3 knockout mice, Piek *et al.* (24) reported that Smad2 and Smad3 mediated the transcription of distinct genes in fibroblasts stimulated by TGF-β. Indeed, these cell systems are useful for comparing the functions of Smad2 and Smad3 in cells derived from embryos of knockout mice. However, because Smad2 knockout is lethal to embryonic mice because of embryo development defects, it had been difficult to examine specific roles of Smad2 and Smad3 concurrently in fully differentiated cells derived from matured organs. In the present study, however, we have demonstrated specific roles of Smad2 and Smad3 in TGF-β_1 regulation of PSC functions isolated from the mature pancreas by employing adenovirus-mediated co-expression of Smad2 or Smad3 with dominant-negative Smad2/3. It is noteworthy that this method can be widely applied for the study on TGF-β intracellular signaling pathway in a variety of mature organs because adenovirus-mediated gene transfer is highly effective in various cell types.

Our present observations that TGF-β_1 activates PSCs through a Smad2-dependent pathway and inhibits PSCs proliferation through a Smad3-dependent pathway provide a novel therapeutic strategy for pancreatic fibrosis. Because TGF-β_1 is a key activator of PSCs (7) and a main inducer of pancreatic fibrosis (4), the therapeutic effect of TGF-β_1 stimulus inhibition on pancreatic fibrosis has been extensively studied. For example, Menke *et al.* (5) reported that inhibition of TGF-β_1 by injection of neutralizing TGF-β_1 antibody reduced extracellular matrix formation in pancreatitis *in vivo*. However, TGF-β_1 is also an autocrine inhibitor of PSC proliferation (6). Thus, blockade of TGF-β_1 activity promotes PSC proliferation. If TGF-β_1 stimuli on PSC activation could be selectively blocked without diminishing the TGF-β_1 inhibitory effect on PSC proliferation, it could be a more potent therapeutic method for pancreatic fibrosis. In this respect, our present data indicate that selective blockade of the Smad2-dependent pathway without affecting the Smad3-dependent pathway can be a novel strategy for the treatment of pancreatic fibrosis .

Our data on TGF-β_1 mRNA expression and peptide secretion of PSCs are important. Because TGF-β_1 mRNA expression and TGF-β_1 peptide secretion of PSCs were not affected by overexpression of Smad2, Smad3, or dominant-negative Smad2/3, we could apply their overexpression and co-expression to observe Smad2- and Smad3-specific roles in the regulation of PSC function by autocrine TGF-β_1. We also demonstrated that TGF-β_1 enhanced its own mRNA expression through a Smad-independent but ERK-dependent pathway. To date, TGF-β_1 has been shown to augment the expression of its own mRNA (25) in both normal and transformed cells, and the promoter sequences of TGF-β_1 gene responsive to the autoinduction have been identified (26). In addition, Yue and Mulder (27) reported that MEK-ERK pathway activation is required for TGF-β_1 expression induced by TGF-β_3, a TGF-β isoform derived from a gene distinct from that of TGF-β_1. However, the intracellular signaling pathway of TGF-β_1 autoinduction has never been demonstrated. Thus, to our knowledge, this is the first report that has elucidated the intracellular signaling pathway of TGF-β_1 autoinduction.

In conclusion, we showed that TGF-β_1 regulates PSC activation, proliferation, and TGF-β_1 mRNA expression through Smad2-, Smad3-, and ERK-dependent pathways, respectively. These observations provide new insights for understanding the mechanism of pancreatic fibrosis and developing a novel therapeutic strategy for its treatment.

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Angiotensin II stimulates DNA synthesis of rat pancreatic stellate cells by activating ERK through EGF receptor transactivation☆

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Abstract

Although angiotensin II (Ang II) is known to participate in pancreatic fibrosis, little is known as to the mechanism by which Ang II promotes pancreatic fibrosis. To elucidate the mechanism, we examined the action of Ang II on the proliferation of rat pancreatic stellate cells (PSCs) that play central roles in pancreatic fibrosis. Immunocytochemistry and Western blotting demonstrated that both Ang II type 1 and type 2 receptors were expressed in PSCs. [^{3}H]Thymidine incorporation assay revealed that Ang II enhanced DNA synthesis in PSCs, which was blocked by Ang II type 1 receptor antagonist losartan. Western blotting using anti-phospho-epidermal growth factor (EGF) receptor and anti-phospho-extracellular signal regulated kinase (ERK) antibodies showed that Ang II-activated EGF receptor and ERK. Both EGF receptor kinase inhibitor AG1478 and MEK1 inhibitor PD98059 attenuated ERK activation and DNA synthesis enhanced by Ang II. These results indicate that Ang II stimulates PSC proliferation through EGF receptor transactivation—ERK activation pathway.

Keywords: Angiotensin II; Pancreatic stellate cell; Pancreatic fibrosis; Epidermal growth factor receptor; Transactivation; Extracellular signal regulated kinase

Angiotensin II (Ang II) is an octapeptide that exerts diverse physiological and pathological actions on cardiovascular systems. It physiologically regulates blood pressure, aldosterone secretion, and salts and potassium homeostasis by acting on vascular smooth muscle, kidney, and adrenal gland [1]. Ang II also acts as a growth factor of myocytes and myofibroblasts in pathological conditions such as remodeling and fibrosis of the heart after chronic hypertension and myocardial infarctions [2]. Besides the action on cardiovascular systems, Ang II has been recently revealed to play important roles in extra-cardiovascular organs. For example, angiotensin II is localized to brain and regulates neurotransmitters release [3]. Ang II also functions in reproductive systems. Ang II mediates electrolyte and fluid secretion of epididymis [4], and also regulates ovarian steroidogenesis such as estrogen [5]. In addition to the various physiological actions, Ang II participates in tissue repair and fibrogenesis of extra-cardiovascular organs. Ang II promotes pulmonary fibrosis after lung injury [6] and also mediates hepatic fibrosis after chronic liver injury [7].

In pancreas, the presence of rennin–angiotensin system has been recently demonstrated. Both angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors are immunocytochemically localized to pancreatic ductal and acinar cells [8]. Moreover, expression of both rennin and Ang II precursor angiotensinogen has been also demonstrated

☆ *Abbreviations:* Ang II, angiotensin II; ERK, extracellular signal regulated kinase; MEK, mitogen-activated protein kinase kinase; PSC, pancreatic stellate cell; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; GPCR, GTP-binding protein coupled receptor; α-SMA, α-smooth muscle actin.

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in pancreas [9,10]. These recent observations suggest that Ang II may physiologically regulate pancreatic functions. More recently, Ang II has been also suggested to play pathophysiological roles in acute and chronic pancreatic injury. Leung et al. [11] reported that experimentally induced acute pancreatitis caused significantly increased expression of angiotensinogen and both AT_1 and AT_2 receptors in pancreas. Their group further reported that blockade of Ang II receptors attenuated pancreatic tissue injury in experimental acute pancreatitis [12]. As to chronic pancreatic injury, Chan et al. [13] reported that chronic hypoxia markedly enhanced expression of angiotensinogen, AT_1 and AT_2 receptors in pancreas, suggesting the possible participation of Ang II in pancreatic chronic injury. In this respect, Kuno et al. [14] demonstrated that angiotensin converting enzyme inhibitor attenuated pancreatic fibrosis in vivo, indicating that angiotensin II promotes pancreatic fibrosis. However, the precise mechanism of Ang II promoting action on pancreatic fibrosis is still unknown.

Pancreatic stellate cells (PSCs) are recently identified, isolated, and characterized [15,16]. In the normal pancreas, PSCs possess fat droplets containing vitamin A and are quiescently defined with desmin positive but α-smooth muscle actin (α-SMA) negative staining [16]. When cultured in vitro, PSCs are auto-activated (auto-transformed) changing their morphological and functional features [15]. PSCs commence losing vitamin A containing lipid droplets, highly proliferating, increasing expression of α-SMA, and producing and secreting extracellular matrix components such as collagen and fibronectin. Namely, PSCs are auto-transformed to myofibroblast-like cells. In vivo, PSCs are also activated during both human and experimental pancreatic fibrosis [17]. Therefore, PSCs are believed to play an important role in pancreatic fibrogenesis. Since Ang II has been suggested to participate in pancreatic fibrogenesis as described above, we hypothesized that Ang II may modulate PSC functions and consequently promote pancreatic fibrosis. We thus conducted the present study to examine the effect of Ang II on PSCs. We report here that Ang II enhances DNA synthesis in PSCs through AT_1 receptor. Experiments are then expanded elucidating the intracellular molecular mechanism of Ang II enhancement of DNA synthesis in PSCs. Our results indicate that Ang II increased DNA synthesis in PSCs by activating ERK through EGF receptor transactivation.

Materials and methods

Reagents. Recombinant human Ang II was purchased from Peptide Institute (Osaka, Japan). Pronase, Nycodenz, and PD123319 were from Sigma (St. Louis, Missouri, USA). DNase I was from Roche (Basel, Switzerland). Collagenase P was from Boehringer–Mannheim (Mannheim, Germany). AG1478 and PD98059 were from Calbiochem (San Diego, California, USA). Anti-AT_1 rabbit polyclonal, anti-AT_2 rabbit polyclonal, and anti-tyrosine-phospho-ERK mouse monoclonal antibodies were from Santa Cruz (Santa Cruz, California, USA). Anti-ERK rabbit polyclonal antibody was from Cell Signaling (Beverly, MA, USA). HRP-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG, and Cy3-conjugated donkey anti-rabbit IgG antibodies were from Jackson Immuno Research (West Grove, Pennsylvania, USA). Losartan is a gift from Banyu Pharmaceutical (Tokyo, Japan).

Isolation and culture of rat pancreatic stellate cells. Rat pancreatic stellate cells were prepared as described [16]. Briefly, rat pancreas was digested in Gey's balanced salt solution supplemented with 0.05% collagenase P, 0.02% pronase, and 0.1% DNase I. After filtration through nylon mesh, cells were centrifuged in a 13.2% Nycodenz gradient at 1400*g* for 20 min. PSCs in the band just above the interface of the Nycodenz solution and the aqueous one were collected, washed, and resuspended in Iscove's modified Dulbecco's medium containing 10 % fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. PSCs were cultured in a 5% CO2 atmosphere at 37 °C. All experiments were carried out using PSCs between passages one and two.

Measurement of DNA synthesis. DNA synthesis was examined by measuring [^{3}H]thymidine incorporation into cells. [^{3}H]Thymidine was added to the culture medium and incubated for 6 h, and [^{3}H]thymidine incorporation was measured as described previously [18].

Western blotting. Western blotting was carried out as described previously [19], using the enhanced chemiluminescence reagent to visualize the secondary antibody. For gel electrophoresis, 10 μg protein was loaded on each lane of a 10% (for Ang II receptors and ERK) or a 7.5% (for EGF receptor) sodium dodecyl sulfate–polyacrylamide gel.

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as described previously [20], using an Olympus BX51 microscope (Olympus Tokyo, Japan). Images were digitized and then processed using Photoshop 5.0 software (Adobe Systems, Mountain View, California, USA).

Statistical analysis. ANOVA was used to determine statistical significance. A value of $P < 0.05$ was considered significant.

Results

AT_1 and AT_2 receptors are present in PSCs

As the first attempt to elucidate Ang II effects on PSCs, we examined the presence of Ang II receptors in PSCs. As shown in Fig. 1A, Western blotting revealed that both AT_1 and AT_2 receptor proteins are expressed in PSCs. Moreover, immunocytochemistry also revealed that both signals of AT_1 and AT_2 receptors are observed in PSCs (Fig. 1B). These signals were abolished when antibodies were preincubated with competing peptides to each antibody (data not shown). These data indicate that AT_1 and AT_2 receptors are present in PSCs.

Ang II increased DNA synthesis in PSCs through Ang II type 1 receptor

We next examined the effect of Ang II on PSC proliferation. To examine PSC proliferation, we determined DNA synthesis in PSCs using [^{3}H]thymidine incorporation assay. As shown in Fig. 2A, Ang II enhanced