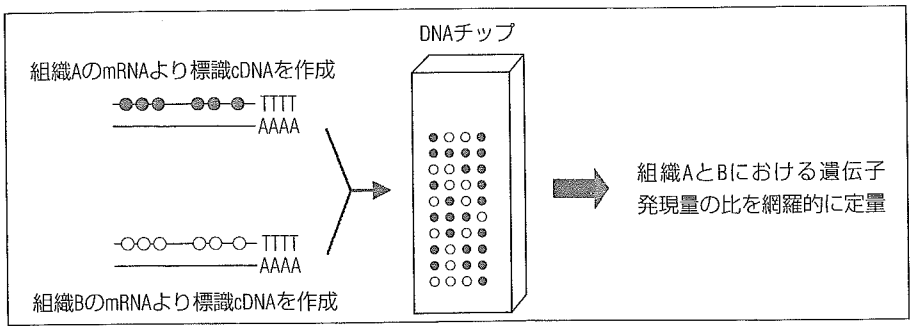

図2 DNAチップ実験の原理

組織AとBとの間で遺伝子発現プロファイルを比較したい場合，まず組織AとBそれぞれから等量のmRNAを調整する．次にこれらmRNAからオリゴdTプライマーと逆転写酵素(RT)を用いてcDNAを合成する．その際に蛍光色素であるCy3あるいはCy5で標識したdUTPをそれぞれに加えることで，各組織由来のcDNAを異なった蛍光色素で標識する．この標識cDNA混合物を，DNAチップとハイブリダイゼーションさせることで，各スポットに標識cDNAが結合する．その結果，任意のスポット上の遺伝子に関する組織Aと組織Bにおける発現量の比は，Cy3とCy5との蛍光強度の比で表されることになる．

しかし，DNAチップがその新しいパワーを最も発揮できる局面は，「疾患の分類」自体を再定義できることではないだろうか．たとえば，多くの癌はその細胞の形態からサブグループが定義されてきたが，DNAチップを用いた解析によって「治療に対する反応性」自体を規定する遺伝子が検出可能であるし，それら遺伝子の発現量に依存した形での疾患の新しい分類法が提唱されるであろう．そうなってこそ初めて，「臨床診断に応用される簡易DNAチップ」が現実のものになると思われる．

遺伝子多型解析

ヒト染色体上には数百塩基対に1つ程度の割合で配列の多様性があり，なかでも1塩基の多型(single nucleotide polymorphism：SNP)が代表的である．ある遺伝子のプロモーター配列上にSNPが存在すれば，その配列に依存した形で遺伝子発現量が変化することが予想される．同様にSNPがエキソン上にあれば，mRNAの安定性や，コードする蛋白質の配列自体に影響を及ぼすこともある．このようなSNPに代表される遺伝子多型は，各個人の生活習慣病への罹りやすさや，薬剤の代謝能力を直接反映していると予想されており，いわば「遺伝的個性」を規定すると考えられる．

多型をゲノムワイドで解析する技術も急速に進歩しており，TaqMan PCR法，Invader法などさまざまな方法が広く用いられている．またSNP解析専用のDNAチップも市販されており，これらの技術を通して，疾患関連SNPの同定が世界中で大規模に試みられている[4]．

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血液・免疫・腫瘍 BIC Forum

別冊

メディカルレビュー社

7　ゲノミクス解析に基づく白血病治療

間野　博行*

Key Sentences

①DNA マイクロアレイによる遺伝子発現プロファイルによって白血病の新しい鑑別診断が可能になる．
②遺伝子発現プロファイルを利用することで，治療反応性を予測可能な新しい疾患グループが定義できる．
③遺伝子多型を解析することで代表的な白血病治療薬の最適量を患者個人に最適化できる．

Key Words

急性骨髄性白血病，悪性リンパ腫，多発性骨髄腫，GST

はじめに

約 30 億塩基対におよぶヒトゲノムの核酸配列を決定する大規模事業である「ヒトゲノムプロジェクト」がついに 2003 年 4 月に終了宣言を行い，ヒト染色体の euchromatin 領域のほぼ完全な塩基配列が決定された (http://www.ncbi.nlm.nih.gov/genome/seq/)．現在これら配列上に遺伝子を割り付ける作業が行われており，ヒトの持つ総遺伝子数はおそらく 3 万種類前後になると予想されている．現段階では遺伝子予測プログラムの精度などの問題から実際の全遺伝子構造の解明にはまだ時間がかかるが，かつて夢であったヒトの全遺伝子プールの全貌がいよいよ明らかになろうとしている．

これからの「ポストゲノム」時代においては，造血器悪性腫瘍の臨床も大きく様変わりすることが予想される．例えば疾患の分類・カテゴリーについても，旧来の診断法に大きく寄与していたものは病理学すなわち「疾

Genomics-based medicine for leukemias

＊　Mano Hiroyuki：自治医科大学ゲノム機能研究部教授

表1　AMLにおける予後関連遺伝子

VDAC1	*ZYX*	*VAT1*	*NPC2*	*AZU1*
ECGF1	*HOMER-3*	*PGD*	*ENSA*	*TKT*
BST1	*STK17B*	*CDK6*	*RAB32*	*PTP4A2*
APLP2	*CYLN2*	*OGT*	*HNRPD*	*POLR2H*
TIAL1	*ATP6F*	*NME1*	*GRIK5*	*CD14*
GABARAP	*NFKBIA*	*GCN5L2*	*HSPE1*	*LBR*
ECE2	*BZRP*	*XPO1*		

（文献5より改変、引用）

患責任細胞の形態学的変化」であった．しかし例えばこれまでの分類では単一の「白血病」と考えられていたものでも，実際の患者1人ひとりの治療反応性は大きく異なる．ある患者では骨髄移植を行っても短期間の内に白血病の再発が生じる一方，別の患者では化学療法で速やかに治癒がもたらされる．今後の医療においては，ゲノミクス技術を駆使した大規模な疾患細胞の情報が蓄積され，これを基にした新たな層別治療が可能になると期待される．各患者の治療反応性の違いを精度良く予測する方法の開発も可能になるであろうし，また例えば特定の薬を使うことによって生じる副作用の種類・程度を予測することも可能ではないであろうか？

新しい疾患の分類法

疾患責任細胞のダイナミックな遺伝子変化をリアルタイムに把握し，疾患の予後に直結した分類法・診断法の開発の上では，DNAマイクロアレイを中心とした網羅的遺伝子発現解析が有用である[1)2)]．例えば白血病の診断を行う場合，旧来では患者骨髄細胞を①ペルオキシダーゼ，エステラーゼなどの特殊染色，②FACSを用いた細胞表面マーカーの解析，③遺伝子異常の有無の解析（免疫グロブリン遺伝子やT細胞表面受容体遺伝子の再構成，*BCR-ABL*遺伝子，*PML-RARα*遺伝子などの有無），④染色体型の解析，などの情報を基にそれぞれの疾患におけるFAB分類を行ってきた[3)]．これらを総合的に解析することで予後にある程度リンクした診断が行えるが，上記の解析を全て行うのは煩雑であり多くの専門技術も必要とする．一方，例えば一枚のDNAマイクロアレイを用いることで旧来の方法で得られる情報がすべて解析可能となれば，診断はより簡便になりかつ異なった施設間での診断の一致率も上昇するであろう．

このようなDNAマイクロアレイを用いた診断の試みとしてGolubらは，6817遺伝子を配置したDNAマイクロアレイによる発現プロファイルに基づいて，急性骨髄性白血病（AML）と急性リンパ性白血病（ALL）とを鑑別する可能性について検討した[4)]．AML 11例，ALL 27例をこのDNAマイクロアレイによって解析した結果，約50個の遺伝子がAMLとALLの間で偏って発現していることが明らかになった．そこでこれら“informative genes”から20個の遺伝子を選びそれらの発現量の偏りの程度を数値化したものに実際の発現量をかけあわせ合計することで，それぞれの患者のAMLあるいはALLの確からしさを数値化した．本法を患者におけるAMLとALLの鑑別診断に応用してみると38例中34例で正しい診断が行われ，1例不明，3例が誤診であった．したがってある程度の確からしさを持ってDNAマイクロアレイによる疾患診断が可能であることがわかった．

一方DNAマイクロアレイによる解析は新しい疾患分類法の開発にも有用である．Yagiらは小児AML患者54例についてAffymetrix社GeneChip HGU 95Aチップを用いた遺伝子発現解析を行い，小児AML内での予後良好群と不良群とを予測する試みを行っている[5)]．彼らのサンプル内で3年以上完全寛解を維持している症例9例と初回緩解導入に失敗した症例9例の間で発現量が統計的に異なる遺伝子35種類を抽出し（**表1**），これら予後関連遺伝子の発現をもとして，患者全体を2 wayクラスタリング法あるいはsupported vector

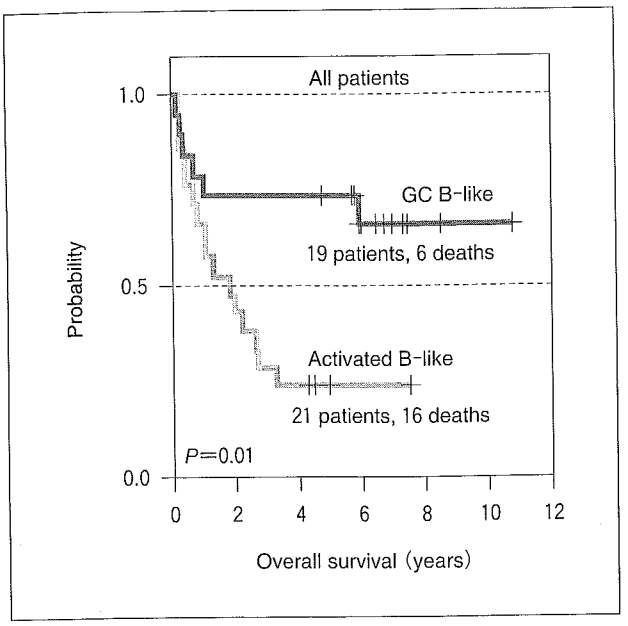

図1　びまん性大細胞型リンパ腫の予後

びまん性大細胞型悪性リンパ腫患者を，リンパ節の遺伝子発現プロファイルから「濾胞中心Bリンパ球に発現パターンが似たサンプル群(GC B-like)」と「活性型Bリンパ球に似た群(Activated B-like)」に分け，両者の生命予後をグラフ(Kaplan-Meier解析)にした．後者が有意に予後不良群であることがわかる．

(文献6より改変，引用)

machine (SVM)法によって層別化した．その結果どちらの方法によってもAML内で生命予後が異なるサブグループを検出することに成功している．すなわち遺伝子発現プロファイルを基にした新しい疾患分類法の可能性が提示されたことになる．

悪性リンパ腫についても，AlizadehらはDNAアレイを用いて非Hodgkinリンパ腫の1つであるびまん性大細胞リンパ腫の新たな予後予測法の開発を試みている[6]．その結果，びまん性大細胞リンパ腫には濾胞中心Bリンパ球に遺伝子発現パターンが似ている群と活性化Bリンパ球に似ている群が存在することが示され，しかも両群間で予後に有意な差が認められることが明らかになった．すなわち活性化Bリンパ球に似た細胞からなるリンパ腫患者の5年生存率(16％)は濾胞中心Bリンパ球に似た細胞からなるリンパ腫患者のそれ(76％)に比べて有意に低いことがわかった(図1)．このことはDNAマイクロアレイによる解析で非Hodgkin悪性リンパ腫の新たなサブグループが定義可能なこと，しかもその分類が予後判定に有意義な情報を与えることを示唆しており，今後の臨床の場におけるDNAマイクロアレイの新たな可能性を示したものとして意義深い．

多発性骨髄腫(multiple myeloma ； MM)はCD 138陽性形質細胞の腫瘍性増殖であるが，ZhanらはMM内の予後良好群と不良群を鑑別する目的で，CD 138に対するアフィニティカラムを用いて同分画を純化しアレイ解析を行っている[7]．具体的に健常者31例，MGUS患者5例およびMM患者74例の骨髄より形質細胞分画を純化し，Affymetrix社のGeneChip HuGeneFLチップ(～6800遺伝子)による実験を行った．これら発現データを基にMMサンプルの系統樹を作成すると，図2に示されるように大きく同患者が4種類のサブグループ(MM 1～MM 4)に別れることが明らかになった．しかもMM 1はmonoclonal gammopathy of undetermined significance (MGUS)に最も近い遺伝子発現プロファイルをもち，一方MM 4グループは骨髄腫細胞株に最も近似したプロファイルをもつことも示された．すなわちMM内のこれらサブタイプが患者の予後にリンクする可能性が示唆されたのである．

遺伝子多型解析

ヒトゲノムの解明が医学・医療にもたらす第二の革新は個人の持つ先天的なゲノムの多様性・個性の解明であろう．ヒトゲノムの配列は各個人間で完全に同じではなく，例えばアングロサクソンとコーカシアンでは人種特異的な塩基配列の違いが存在することが知られる．また例えば同じ日本人の中でも地域によって固有の塩基配列の多型があり，さらに部分的には各家系，個人にも固有の配列多型があると思われる．一塩基多型(single nucleotide polymorphism ； SNP)が話題に上ることが多いが，これらSNPを含む遺伝子配列の多型が個人の遺伝的個性を決定していると予想される．

今後の白血病臨床における遺伝子多型が持つ重要な意義は，薬剤感受性あるいは薬剤の副作用発症の予測である[8][9]．例えばアミノグリコシド系抗生剤の重要な副作

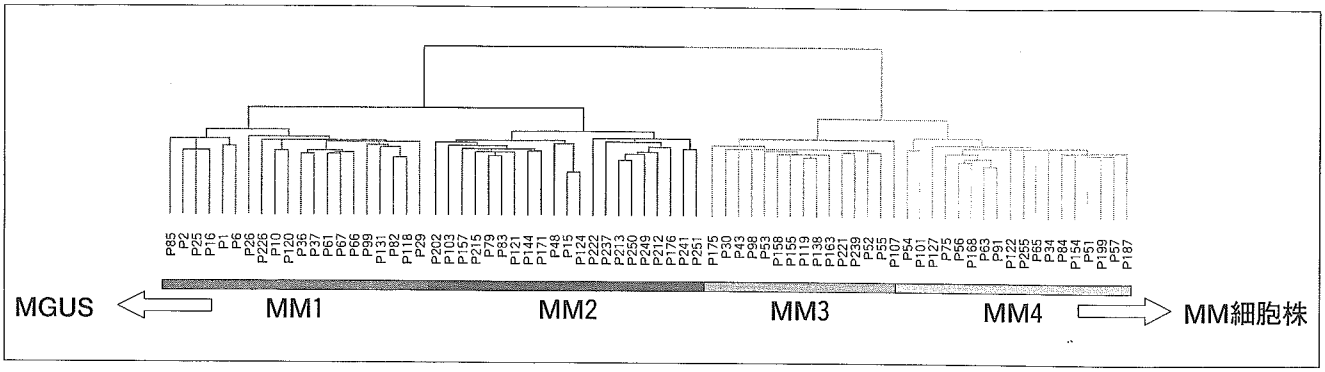

図2　MM患者サンプルの遺伝子発現プロファイルによる系統樹

MM患者74例の骨髄CD 138陽性細胞における遺伝子発現プロファイルを基にサンプルの系統樹を作成したところ，MM 1～MM 4までの4種類のサブタイプに大きく分類されることが示された．なおMM 1はMGUSのCD 138陽性細胞に似た発現プロファイルを持ち，MM 4は骨髄腫細胞株群と似たプロファイルを持つ．

（文献7より改変，引用）

用に難聴があるが，薬剤投与の結果難聴が生じるのはごく一部の患者のみである．詳細な解析の結果12 Sリボゾーム RNA遺伝子にA 1555 G変異があると重度の難聴が発症することがわかっており，このような多型をあらかじめ検査することで副作用の発生を最小限にとどめることが可能になるであろう．

一方有効な抗癌剤を選択する上でも遺伝子多型は重要な評価ポイントとなるであろう．細胞内は常に酸化ストレスにさらされている状態であり，真核生物にはこのような酸化状態から細胞を守る様々なメカニズムが存在する．その代表的なものがグルタチオンを利用した還元系であり，なかでもglutathione S-trasnferase (GST)は重要な役割を担っている[10]．GST活性は重要な抗アポトーシス因子であり，実際ある種の癌細胞においては亢進したGST活性により抗癌剤が無効になる．しかもGST遺伝子ファミリーは遺伝子多型が豊富なことでも知られており，遺伝子の配列異常，欠失を含む変化が抗癌剤感受性を直接制御している．代表的な薬剤感受性・毒性関連遺伝子多型に関する個人情報が入力された電子カードをもって診療を受ける，というような時代も遠からず訪れるのではないだろうか．

最後に

ヒトゲノムの完全版配列が完成しても，そこに存在する遺伝子の多様性はまだ十分に解明されていない．これまで「遺伝子」は暗黙のうちに「蛋白をコードする単位」と考えられていたが，意外にもたとえ長大なmRNAを作っても蛋白をコードする領域を持たない「遺伝子」が極めて多く存在することもわかってきた[11]．また数は少ないもののmicroRNAと呼ばれる20-30塩基の短いRNAも全く異なった生理機能を持って存在しているらしい．ヒトゲノムプロジェクトの成果を医療・社会へフィードバックする事業はまだ始まったばかりである．この分野の進歩においてもわが国から多くの貢献がなされることを期待したい．

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予後の予測

急性白血病

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1．急性白血病

間野博行

急性白血病の治療戦略は，アントラサイクリン＋シトシンアラビノシドを中心とする化学療法と骨髄移植療法とを2本柱としたプロトコールで組み立てられてきた．しかし近年，白血病の病態解明と臨床データが蓄積されるに従い，各患者個人に最適化された治療スケジュールの構築が求められている．急性白血病はきわめて多様な病因・臨床像からなるいわば症候群のようなものであるため，白血病患者への治療法の最適化のためには，白血病の成因・予後因子に応じた形での新たな患者層別化が必要であろう．そのよい例としてt(15;17)を有する急性前骨髄球性白血病（APL）があげられる．この染色体転座の結果，レチノイン酸受容体（RARα）とPMLとの融合蛋白質が産生されるが，本分子を標的としたall-trans retinoic acidはAPLの寛解導入に著効するのである．

これまで急性白血病の分類には，主に白血病細胞の形態学を基盤としたFrench-American-Britishグループ（FAB）分類[1]が利用されてきたが，近年の遺伝子解析の知見を取り入れたWorld Health Organization（WHO）分類が1999年に提唱された[2]．しかしながら，これらの分類法は各患者の予後予測にはいまだ不充分であり，たとえばDNAマイクロアレイによる網羅的発現解析データを取り入れる工夫などが試みられている．

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急性骨髄性白血病（AML）

旧来のFAB分類では，APLに相当するM3サブタイプが予後良好なこと，また未分化なタイプのM0および赤白血病M6，巨核芽球性白血病M7が予後不良なことが知られていた．しかしながら，症例数の多いM1やM4サブタイプの患者予後は均一ではなく，新たな層別化のマーカーが待たれていた．その後，AMLにおいてしばしば観察される染色体転座の原因遺伝子が同定され，これら染色体異常と各患者予後との詳細な解析がなされるに至った．

現段階では，これらの知見を取り入れた核型による患者層別化がシンプルでかつAMLの予後予測に最も有効なものといえる．Medical Research Council（MRC）による1,600例に及ぶAML患者の核型解析の結果，表1に示される患者層別化が

表1　核型に基づくAMLサブグループの定義（文献3より改変）

グループ	核型
favorable	t(8;21) t(15;17) inv(16)
intermediate	all others
adverse	−5/del(5q) −7 abnormal 3q complex

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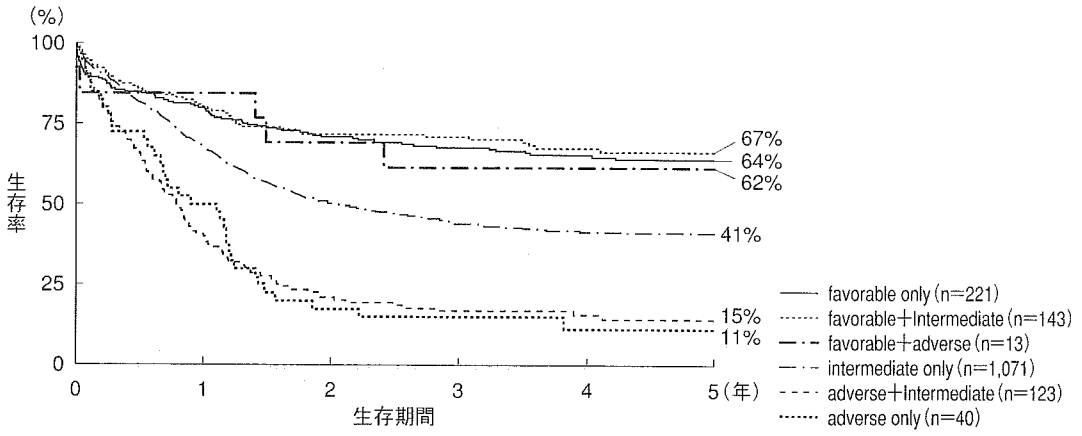

図1　核型による患者生命予後（文献3より改変）
表1に示されるfavorable，intermediate，adoverse各患者グループの生存曲線をKaplan–Meier解析で示す．3群の長期生存率が大きく異なることがわかる．

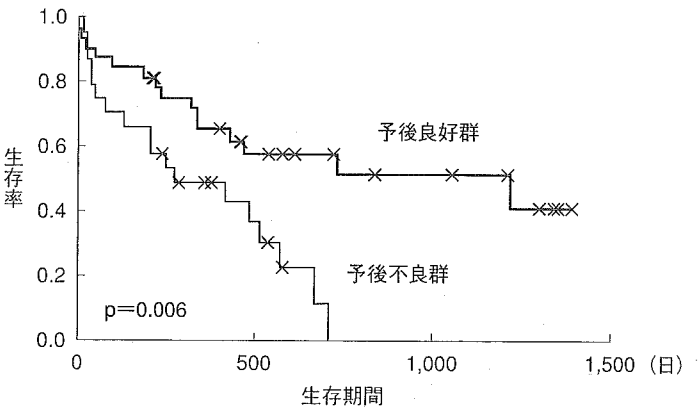

図2　DNAマイクロアレイによる層別化（文献5より改変）
DNAマイクロアレイ解析の結果同定された「予後にリンクする遺伝子セット」の発現量を用いて，患者を2群に分類した．両群間の生存曲線が大きく異なることをKaplan–Meier解析で示す．

どの遺伝子の量的変化が重要なのかは全く不明なままである．これら核型による予後予測はきわめて強力であり，たとえば表1の各グループの長期生命予後を比較すると図1の生存曲線に示されるとおり，3群は大きく異なる予後を有することが明らかである．なお，「正常核型」が予後良好群ではなくintermediate groupに属することは注意すべきであろう．

核型による分類だけでは，AML患者の約半数を占める正常核型を有する患者の層別化が困難である．そこで核型以外のさまざまなパラメータも単変量あるいは多変量解析によって検討されてきた．たとえばJapan Adult Leukemia Study Group（JALSG）の解析では，年齢，芽球のmyeloperoxidase陽性率，performance status，末梢血白血球数，血球の異形成の有無などが生存に有意にリンクすることが報告されている[4]．

近年では，DNAマイクロアレイを用いた網羅

提案された[3]．予後良好なfavorable groupに属するt(15;17)，t(8;21) およびinv(16) は，それぞれFAB分類におけるM3，M2およびM4 Eoに相当する．また，intermediate groupに属する11q23転座はMLL遺伝子の変化を含むことが多い．一方，monosomy 7および5q－は重要な予後不良因子であるが，本核型異常において具体的に

的遺伝子発現データによってAML芽球の遺伝子発現プロファイルをとらえ，そのパターンから予後を予測する試みもなされている．たとえばBullingerらは，26,260種類の遺伝子が配置されたDNAマイクロアレイを用いて，116例のAML検体（骨髄あるいは末梢血単核球）の遺伝子発現データを得た[5]．これらの遺伝子中，患者予後にリンクするもの133種類を抽出し，その発現プロファイルから患者を大きく2群に分けている．その結果図2に示されるように，両患者グループの長期予後は有意に異なることが明らかになった．しかもこれら遺伝子データによる分類は，正常核型の患者内でも予後が異なる2群が存在することを示しており，発現プロファイルによる分類が旧来の核型分類とは異なる情報を与えるといえる．

急性リンパ性白血病（ALL）

小児のALLがきわめて予後良好な白血病であるのに比し，成人のALLは一般に予後不良である．ALLはFAB分類によりL1, L2, L3の3種類に分類されてきた．L3は本邦ではまれなBurkittリンパ腫型であり，実際はL1とL2が大部分を占める．今日の治療において患者の生命予後にL1とL2の区別はリンクしておらず，新しいWHO分類でもL1, L2, L3のサブタイプは却下された．成人ALLの予後予測因子を解析した報告は多くないが，AMLの場合と同様に核型が重要な指標となる．Cancer and Leukemia Group B (CALGB) による256例の解析では，t(9;22), t(4;11), monosomy 7, trisomy 8の存在が長期生存に対する予後不良因子であることが示された[6]．さらに高齢，初診時の白血球数高値，B細胞系芽球なども同様な予後不良因子であるとされている．また，JALSGによる本邦ALL症例の解析でも，t(9;22) の存在と高齢（30歳以上），白血球数高値（3万/mm^{3}以上）が予後不良因子であると報告された[7]．

●おわりに

核型による層別化がきわめて有効なのは，急性白血病が多様な症候群であり，その病因単位に治療法を最適化するべきであることを示唆しているといえよう．白血病の成因が漸次明らかになるに伴い，層別化がさらに細分化されるとともに，各病因に対応した分子標的療法が開発されると期待される．一方，病因の多くが不明な今日においては，それを間接的に評価可能なDNAマイクロアレイ解析が有効なツールとなるのではないだろうか．

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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

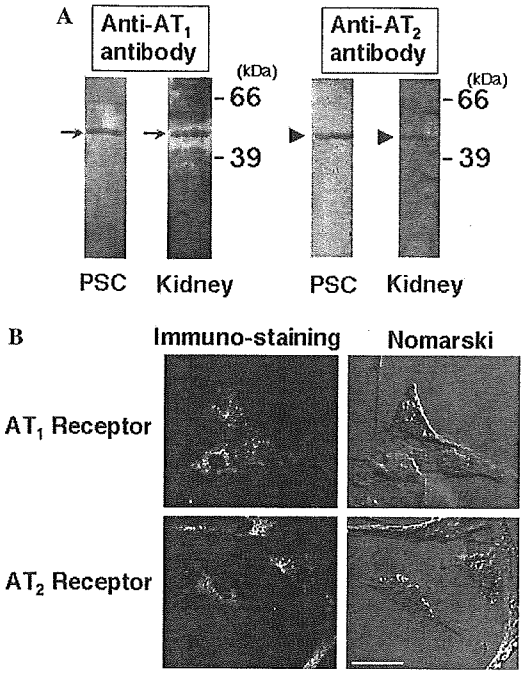

Fig. 1. Presence of AT_1 and AT_2 receptors in PSCs. (A) Western blotting of AT_1 (arrows) and AT_2 (arrow heads) receptors was performed using their specific antibodies. Crude lysate of rat kidney was used as a positive control. Molecular markers are indicated on right. (B) Fluorescence micrographs showing the immunoreactivity of AT_1 and AT_2 receptors in PSCs with corresponding Nomarski images. Immunocytochemistry was carried out with the same first antibodies as those used for Western blotting. Bar: 40 μm.

[^{3}H]thymidine incorporation into PSCs in a dose-dependent manner. The maximum increase was observed at 100 nM. Moreover, losartan, an AT_1 receptor antagonist, inhibited [^{3}H]thymidine incorporation into PSCs enhanced by 100 nM Ang II (Fig. 2B). In contrast, PD123319, an AT_2 receptor antagonist, did not alter [^{3}H]thymidine incorporation into PSCs enhanced by 100 nM Ang II (Fig. 2B). These data indicate that Ang II enhances DNA synthesis in PSCs through AT_1 receptor.

EGF receptor mediates Ang II enhancement of DNA synthesis in PSCs

Ang II receptors are coupled to GTP-binding proteins and possess seven transmembrane domains. Recently, EGF receptor transactivation by GTP-binding protein coupled receptors (GPCRs) has been suggested to be one of the major signaling pathways through which various GPCR-ligands exert their growth-promoting effect on various types of cells [21,22]. We thus hypothesized that Ang II may enhance PSC DNA synthesis through EGF receptor transactivation. To examine this hypothesis, we investigated whether Ang II activates EGF receptor in PSCs. As shown in Fig. 3A, Ang II phosphorylated EGF receptor at its tyrosine

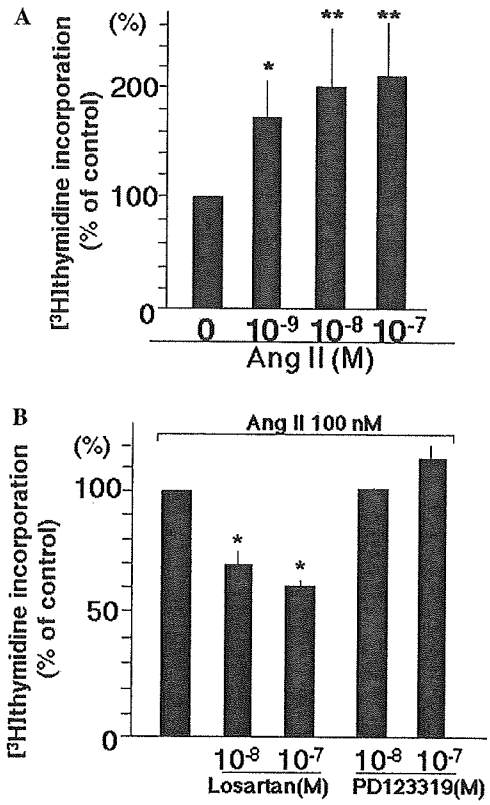

Fig. 2. (A) Effect of Ang II on DNA synthesis in PSCs. Cells were incubated for 48 h with the indicated amounts of Ang II. DNA synthesis was examined by [^{3}H]thymidine incorporation assay. Results are expressed as percent [^{3}H]thymidine incorporation of controls. Values are means ± SEM. The result is representative of four independent experiments with similar results performed in triplicate. (B) Effect of Ang II receptor antagonists on DNA synthesis enhanced by Ang II in PSCs. After 2 h pre-incubation in the presence (losartan; the second and third columns, PD1213319; the fourth and fifth columns) or absence (the first column) of indicated amounts of Ang II receptor antagonists, cells are stimulated for 48 h with 100 nM Ang II. DNA synthesis was examined by [^{3}H]thymidine incorporation assay. Results are expressed as percent [^{3}H]thymidine incorporation of controls. Values are means ± SEM. The result is representative of four independent experiments with similar results performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$.

residue indicating that Ang II activates EGF receptor. EGF receptor phosphorylation by Ang II was observed at 5 min incubation and maximum phosphorylation was observed at 30 min incubation. Moreover, Ang II promoting effect on PSC DNA synthesis was markedly attenuated by PSC pretreatment with EGF receptor kinase inhibitor AG1478 (Fig. 3B). These data imply that Ang II enhances DNA synthesis in PSCs, at least in part, by transactivating EGF receptor.

Ang II activates ERK through EGF receptor transactivation

We next examined whether Ang II activates ERK, which is downstream of EGF receptor-mediated signaling pathway of cellular growth-promoting stimuli.

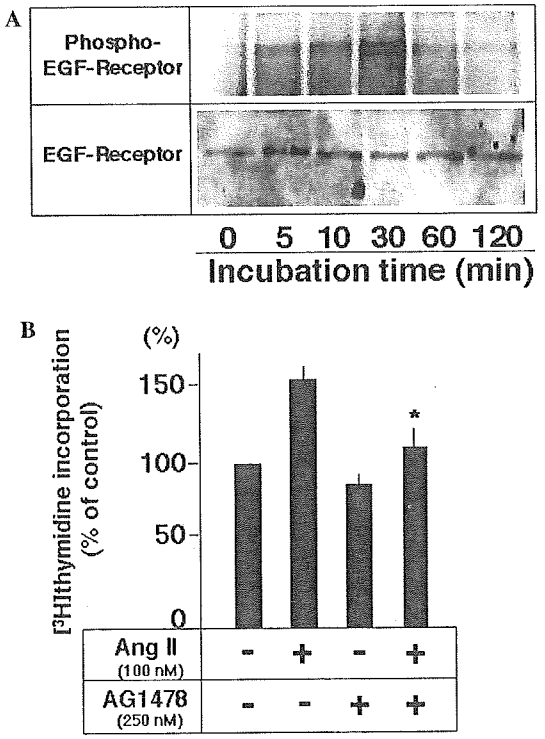

Fig. 3. (A) Effect of Ang II on EGF receptor activation in PSCs. Cells were incubated with 100 nM Ang II for indicated times. The activation of EGF receptor was then determined by Western blotting using anti-phosphorylated EGF receptor antibody (upper panel). Western blotting with anti-EGF receptor antibody (lower panel) was carried out as an internal control. (B) Effect of EGF receptor kinase inhibitor AG1468 on DNA synthesis stimulated with Ang II in PSCs. After 30 min pretreatment with (columns 3 and 4) or without (columns 1 and 2) 250 nM AG1478, cultured PSCs were incubated for 48 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM Ang II. DNA synthesis was examined by [^{3}H]thymidine incorporation assay. Results are expressed as percent [^{3}H]thymidine incorporation of controls. Values are means ± SEM. The result is representative of four independent experiments with similar results performed in triplicate. *$P < 0.05$ vs. the second column.

As shown in Fig. 4A, 100 nM Ang II phosphorylated ERK at its tyrosine residue in PSCs, indicating that Ang II activates ERK. Maximum activating effect was observed at 5–10 min incubation. When EGF receptor kinase activity was blocked with AG1478, Ang II failed to activate ERK (Fig. 4B). These data indicate that Ang II activates ERK through EGF receptor transactivation.

Ang II enhances DNA synthesis in PSCs through ERK activation

Knowing that Ang II activates ERK through EGF receptor transactivation, we finally examined whether activated ERK mediates Ang II promoting effect on DNA synthesis in PSCs. For this purpose, we blocked ERK activation by using the MEK1 inhibitor PD98059. As shown in Fig. 5A, pretreatment of PSCs with PD98059 blocked ERK activation by Ang II. Furthermore, Ang II promoting effect on DNA synthesis in

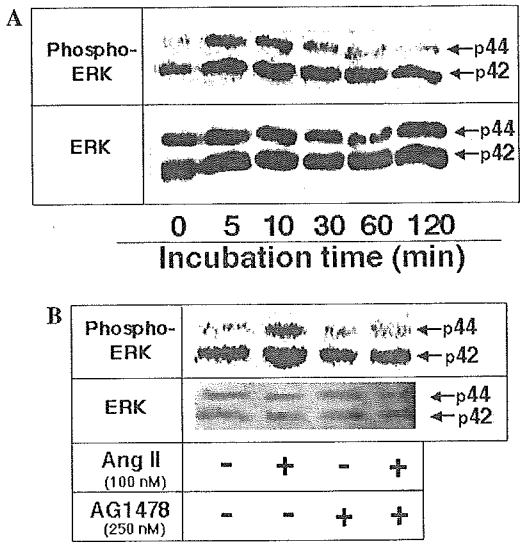

Fig. 4. (A) Effect of Ang II on ERK activation in PSCs. Cells were incubated with 100 nM Ang II for indicated times. The activation of ERK was then determined by Western blotting using anti-phosphorylated ERK antibody (upper panel). Western blotting with anti-ERK antibody (lower panel) was carried out as an internal control. (B) Effect of EGF receptor kinase inhibitor AG1468 on ERK activation stimulated with Ang II in PSCs. After 30 min pretreatment with (lanes 3 and 4) or without (lanes 1 and 2) 250 nM AG1478, cultured PSCs were incubated for 5 min in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM Ang II. The activation of ERK was then determined by Western blotting using anti-phosphorylated ERK antibody (upper panel). Western blotting with anti-ERK antibody (lower panel) was carried out as an internal control.

PSCs was also attenuated by PSC pretreatment with PD98059 (Fig. 5B). These data indicate that Ang II enhances DNA synthesis in PSCs by activating ERK through EGF receptor transactivation.

Discussion

In this study, we demonstrated that Ang II enhances PSC proliferation through AT_1 receptor. We further elucidated that Ang II augments PSC proliferation by activating ERK through EGF receptor transactivation. These data suggest the participation of Ang II in pancreatic fibrosis by increasing PSC proliferation.

In addition to the traditional action on blood pressure homeostasis, much attention has been directed to Ang II participation in tissue fibrosis. Ang II acts as a growth factor of cardiac fibroblasts (myofibroblasts) and contributes to cardiac remodeling with fibrosis and hypertrophy [23]. In kidney, Ang II induces the proliferation of mesangial cells and fibroblasts, and consequently promotes renal fibrosis [24]. Ang II is also mitogenic for lung fibroblasts and plays a role in pulmonary fibrosis [6]. As to pancreatic fibrosis, Kuno et al. recently reported that angiotensin-converting enzyme inhibitor attenuated pancreatic fibrosis and decreased the number of

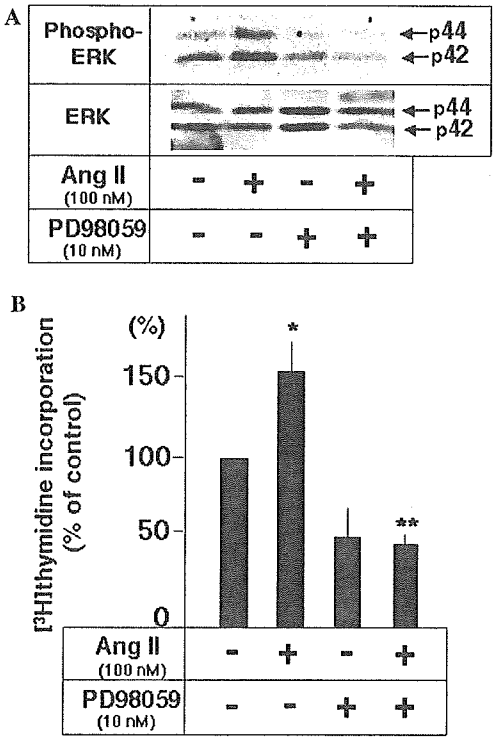

Fig. 5. (A) Effect of MEK inhibitor PD98059 on ERK activation stimulated with Ang II in PSCs. After 2 h pretreatment with (columns 3 and 4) or without (columns 1 and 2) 10 nM PD98059, cultured PSCs were incubated for 5 min in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM Ang II. ERK activation was then determined by Western blotting using anti-phosphorylated ERK antibody (upper panel). Western blotting with anti-ERK antibody (lower panel) was carried out as an internal control. (B) Effect of MEK inhibitor PD98059 on DNA synthesis stimulated with Ang II in PSCs. After 2 h pretreatment with (columns 3 and 4) or without (columns 1 and 2) 10 nM PD98059, cultured PSCs were incubated for 48 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM Ang II. DNA synthesis was examined by [^{3}H]thymidine incorporation assay. Results are expressed as percent [^{3}H]thymidine incorporation of controls. Values are means ± SEM. The result is representative of four independent experiments with similar results performed in triplicate. $P < 0.05$ vs. control, *NS vs. the third column.

activated PSCs in spontaneously occurring chronic pancreatitis in vivo [14]. Since PSCs play a major role in pancreatic fibrosis by synthesizing and secreting various extracellular matrixes such as collagen and fibronectin, their observations suggest the possibility that Ang II may modulate PSC functions. However, its direct evidence had not been demonstrated. Thus, to our knowledge, this is the first evidence of the Ang II regulation of PSCs.

The mechanism of digestive organ fibrosis has been extensively studied on hepatic fibrosis, including Ang II participation. Jonsson et al. [7] revealed that hepatic fibrosis was attenuated by the inhibition of angiotensin-converting enzyme in vivo. Furthermore, Bataller et al. [25] reported that exogenous Ang II induces HSC proliferation in vitro. Taken together, Ang II is assumed to promote hepatic fibrosis by inducing HSC proliferation. Although these reports are consistent with our present data on Ang II action on PSCs, the molecular mechanism of intracellular signaling of Ang II mitogenic effect on HSCs has been still unclear. Thus, we have expanded their studies by elucidating the molecular mechanism of Ang II intracellular signaling in PSCs. We have demonstrated that Ang II activates ERK through EGF receptor transactivation and consequently enhanced PSC proliferation. Warranted is the further study to examine whether similar molecular mechanism underlies the Ang II mitogenic action on HSCs.

Although our current observation with EGF receptor kinase inhibitor AG1478 strongly indicates that Ang II promotes PSC proliferation through EGF receptor transactivation, AG1478 could not completely abolish Ang II enhancement of DNA synthesis in PSCs (Fig. 3B). This might be attributed to the existence of another intracellular signaling pathway of Ang II mitogenic action on PSCs besides EGF receptor transactivation. In this respect, the interplays between GPCRs and TGF-β family signaling have been recently reported. For instance, activin A, a member of TGF-β family, functions as an autocrine inhibitor of DNA synthesis in hepatocytes [26]. We have reported that norepinephrine, a representative GPCR ligand, enhances hepatocyte proliferation by inhibiting activin A signaling with the induction of inhibitory Smad 7 [18]. Since both activin A and TGF-β are also autocrine inhibitors of PSC proliferation [27,28], it is an intriguing open question whether Ang II signaling pathway interacts with TGF-β family signaling in PSCs.

In contrast to EGF receptor kinase inhibitor, MEK inhibitor PD98059 completely abolished Ang II promoting effect on DNA synthesis in PSCs (compare the third and fourth columns in Fig. 5B). This observation suggests that ERK is the major intracellular mediator of Ang II promoting effect on PSC proliferation. Moreover, PD98059 markedly attenuated even basal DNA synthesis in PSCs (compare the first and third columns in Fig. 5B). Since ERK is also a key mediator of mitogenic signals of other growth factors [29], this phenomenon may be attributed to the inhibition of mitogenic effects of other growth factors contained in culture medium used in the present study.

Using Western blotting and immunocytochemsitry, we demonstrated that both AT_1 and AT_2 receptors are present in PSCs (Fig. 1). However, Ang II enhancement of DNA synthesis in PSCs was inhibited by AT_1 receptor blocker losartan, but not by AT_2 receptor blocker PD123319, indicating that AT_1 receptor mediates Ang II stimulation on PSC growth (Fig. 2B). Consistent with our observation, most of Ang II actions well described to date such as vasoconstriction, stimulation of aldosterone release, and promotion of cardiovascular cellular growth are all medicated by AT_1 receptor [30]. As to AT_2 receptor, however, its specific functions have been recently described. AT_2 receptor mediates anti-proliferative

effects in cultured coronary endothelial cells [30,31] and vascular smooth muscle cells [30,32]. Furthermore, AT_2 receptor promotes differentiation in some types of cells [33,34]. Since PSC activation is a kind of differentiation to myofibroblast-like cells and is a cellular function opposite to proliferation, one might speculate that AT_2 receptor could mediate PSC activation. However, Ang II did not exert any effect on PSC activation (Our unpublished data determined with Western blotting using anti-α-SMA antibody). Thus, further studies are needed to elucidate AT_2 receptor function in PSCs.

In conclusion, we have shown that Ang II stimulates PSC proliferation by activating ERK through EGF receptor transactivation. These observations provide new insights into understanding the molecular mechanism of pancreatic fibrosis.

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Aberrant Expression of CDX2 in the Metaplastic Epithelium and Inflammatory Mucosa of the Gallbladder

To the Editor:

We read with great interest the article by Phillips et al, published in the November 2003 issue of the *American* AU1 *Journal of Surgical Pathology*.[6] In this report, the authors mentioned that CDX2 protein was a sensitive marker of intestinal metaplasia in the esophagus. They also stated the usefulness of CDX2 for the detection of histologically equivocal cases of Barrett's esophagus, consistent with our previous reports on the aberrant expression of CDX2 in the Barrett's epithelium as well as inflammatory esophageal mucosal cells.[3,4] Chronic inflammatory mucosa of the gallbladder is often accompanied by the intestinal metaplasia, and there are considerable data suggesting associations between intestinal metaplasia and hyperplasia or dysplasia in the gallbladder. Yamagiwa et al suggested by immunohistochemistry that intestinal metaplasia was often associated with dysplasia and carcinoma in the gallbladder.[7] Other reports also support these relationship by showing intestinal differentiation of dysplastic epithelium within or adjacent to invasive cancer of the gallbladder.[1,2] Despite the importance of elucidating the mechanisms involved in the transition of inflammatory mucosal cells to intestinal metaplasia in the gallbladder, genetic events predisposing to metaplastic changes in the gallbladder are not well documented.

We assessed the expression of CDX2 in the inflammatory mucosal epithelial cells as well as intestinal metaplasia of the gallbladder. Immunohistochemical study demonstrated extensively fine granular immunoreactivity for CDX2 in the cytoplasm of inflammatory mucosal cells in the gallbladder. By contrast, nuclear stainings were observed in the intestinal metaplasia of the gall- F1 bladder (Fig. 1). We have reported that CDX2, expressed in the gastric mucosa of chronic gastritis without intestinal metaplasia, might be a trigger of metaplastic transition in the stomach.[3] We have also reported that intestinal metaplasia was induced in a transgenic mouse overexpressing CDX2 in the stomach.[5] Interestingly, CDX2 was positive even in the epithelium of gallbladder with minimal inflammatory changes. Furthermore, similar to the Barrett's esophagus or gastric intestinal metaplasia, the expression of CDX2 was often observed even in the absence of MUC2 in the inflammatory mucosal cells of the gallbladder, supporting the notion that CDX2 was a marker of epithelial differentiation in the gallbladder. Taken together, these results reinforce the hypothesis that CDX2 expression may play a critical role in the development of intestinal metaplasia in the stomach, esophagus, and gallbladder.

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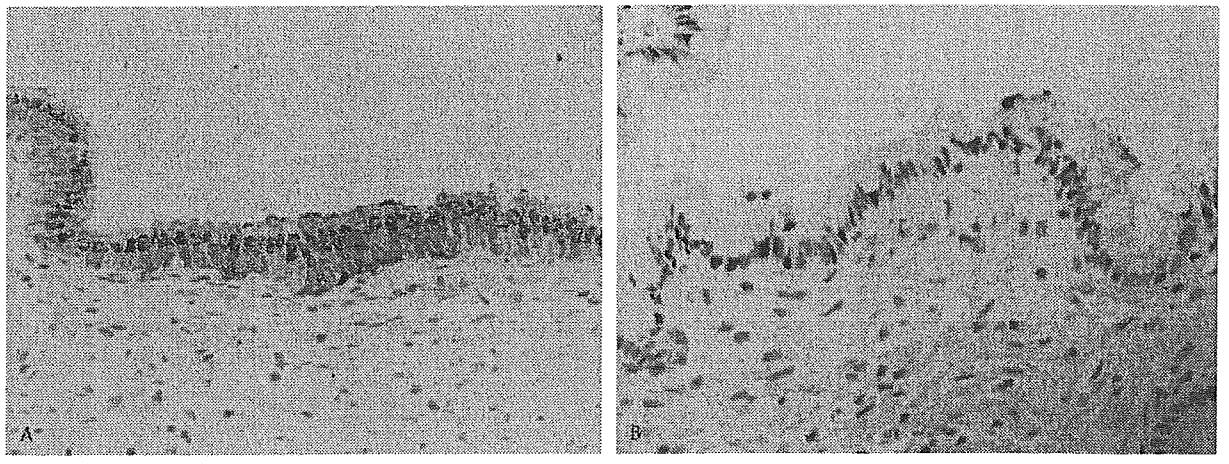

FIGURE 1. Immunostaining of the gallbladder epithelium. Fine granular pattern of cytoplasmic staining in the inflammatory mucosal epithelium (A) and nuclear immunoreactivity in the intestinal metaplasia (B) were observed, respectively (original magnification ×200).

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