Growth factor and hypoxic signaling
Convergence of signaling pathways driven by growth factors and hypoxia has been well described (Pouyssegur *et al.*, 2006). Activation of growth factor receptors leads to the augmentation of HIF expression, which subsequently amplifies expression of VEGF. Even more rigid coupling is seen in hematopoietic stem cells; these cells are unable to express HIF-1α mRNA in the absence of growth factor receptor activation, and growth factor-dependent HIF-1α is involved in the determination of intracellular glucose fate (Lum *et al.*, 2007). Such observations suggest that HIFs cooperate with growth factor signaling in the governance of cellular metabolism.

We observed a self-renewing mitogenic effect of reduced oxygen on glioma CSCs. To investigate the presence and significance of the cross talk between hypoxic signaling and growth factor signaling in glioma CSCs, we interrogated the PI3K and MAPK signaling mechanisms. As reported earlier by other groups, we found that hypoxia was capable of enhancing the activation of growth factor signaling pathways (Alvarez-Tejado *et al.*, 2001; Xu *et al.*, 2004). Furthermore, blocking these pathways resulted in the attenuation of hypoxic induction of HIF-1α by glioma CSCs. It was shown earlier that PI3K pathway activation was neither required nor sufficient by itself for HIF-1α-dependent gene transcription (Arsham *et al.*, 2002). Our experience with glioma-derived CSCs suggests that, indeed, the PI3K pathway activation is not required for HIF-1α induction, but growth factor signaling may amplify such induction under hypoxic conditions. Such observations suggest that specific cellular response to hypoxia is tissue or cell-type specific. Strict dependence of glioma cells on growth factor signaling pathways may link the hypoxia and these pathways more intimately in gliomas.

Our findings indicate that hypoxia contributes to glioma tumor growth by enhanced self-renewal activity and maintenance of the undifferentiated state of a subset of the CSC populations. It further suggests a context-dependent regulation of the tumor-initiating CSC phenotype. Interestingly, growth factor signaling pathways only partially overlap with hypoxia-mediated signaling response. This suggests the importance of fully characterizing the hypoxia-signaling mechanisms in glioma-derived CSCs, because targeting both the hypoxia-growth factor pathway and the hypoxia-specific signaling cascade may provide improved therapeutic opportunities for the treatment of malignant gliomas.

Materials and methods

Tissue specimen
Three CSC lines were established from acutely resected human tumor tissues. The X01 line was derived from a woman with a glioblastoma multiforme. X02 line originated from a man with glioblastoma multiforme. X03 was derived from a woman with anaplastic oligoastrocytoma.

Cell culture
Tumor-sphere cultures were performed as described earlier, with some modifications, in medium containing Dulbecco's modified Eagle's medium-F12 (Gibco-Invitrogen, La Jolla, CA, USA), penicillin G, streptomycin sulfate, B-27 (Gibco-Invitrogen), and recombinant human EGF (20 ng/ml; R&D Systems, Minneapolis, MN, USA) (Singh *et al.*, 2004; Oka *et al.*, 2007; Soeda *et al.*, 2008). Cells were cultured in HERAcell incubators (Thermo Electronic Corporation, Asheville, NC, USA) at 37 °C, 95% relative humidity, and 5% CO_2 with 20% oxygen or 1% oxygen conditions.

Fluorescence-activated cell sorting analysis
To characterize the effects of hypoxia on the CD133-positive CSC sub-population propagated as spheres, $1x10^6$ cells were placed in a proliferation medium containing EGF. After 12–72 h, cells were evaluated on a Coulter EPICS cytometer (Beckman Coulter, Fullerton, CA, USA). To further characterize the effect of hypoxia on CSCs, each sample was labeled with phycoerythrin-conjugated anti-human CXCR4, CD44, CD24 (BD Biosciences, San Jose, CA, USA), phycoerythrin-conjugated CD133/1 (AC133) (Miltenyi Biotec, Auburn, CA, USA), or A2B5 (Miltenyi Biotec) with phycoerythrin-secondary antibody (BD Biosciences) according to the manufacturer's recommendation. Appropriate compensation and isotype controls were used. All experiments were performed in triplicates.

Western blotting
Western blot analyses were performed as described earlier (Soeda *et al.*, 2008). The following antibodies were used: CD133 (Cell Signaling Technology, Beverly, MA, USA), HIF-1α (BD Biosciences), HIF2-α (Novus Biologicals, Littleton, CO, USA), actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-EGFR (P-Tyr1068, Cell Signaling Technology), Akt (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), phospho-ERK1/2 (P-Thr202/Tyr204, Cell Signaling Technology), phospho-p70S6 kinase (Thr-389, Cell Signaling Technology), PHD2 (Cell Signaling Technology), and Notch1 (Cell Signaling Technology). Briefly, tumor spheres were lysed in a buffer consisting of 20 mM Tris-HCl (pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). After brief sonication, lysates were clarified by centrifugation at $12\,000 \times g$ for 10 min at 4 °C, and protein content in the supernatant was measured according to the Bradford method. Aliquots (30–50 μg of protein per lane) of total protein were separated by 7.5–15% SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose transfer membranes (0.2 μm; Amersham Biosciences, Buckinghamshire, UK). Each membrane was blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.01% Tween-20) for 1 h at room temperature, followed by incubation with the appropriate primary antibodies overnight at 4° C. After extensive washing with TBS-T, each membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (1:1000) for 1 h at room temperature in TBS-T containing 5% non-fat dry milk. Detection was performed using an enhanced chemiluminescence reagent (Amersham Biosciences), according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay
Vascular endothelial growth factor protein levels were determined by enzyme-linked immunosorbent assay performed with Quantikine immunoassay for human VEGF (R&D systems) according to the manufacturer's instructions. CSCs

(10^6) were dissociated into single cells and transferred to T75 Falcon culture flasks with suspension medium containing EGF at different oxygen tensions. After 72 h incubations, supernatants were used immediately or frozen at −20° C until they were processed. All experiments were performed in triplicates.

Indirect immunofluorescence microscopy
Immunocytochemistry of CSCs was performed as described (Oka *et al.*, 2007; Park *et al.*, 2007). Primary antibodies used were as follows: anti-β-III-tubulin (Tuj1; mouse mAb, 1:200; Chemicon, Temecula, CA, USA) and anti-glial fibrillary acidic protein (GFAP; rabbit pAb, 1:500; DAKO, Glostrup, Denmark). Alexa Fluor 488 and 555 secondary antibodies were used (1:1000; Molecular Probes, Eugene, OR, USA). Cells were counterstained with 4′,6-diamidino-2-phenylindole. The following hardware was used: Zeiss Axiovert 200 microscope (Carl Zeiss, Gottingen, Germany), Plan-Neofluar ×20 and ×40 objectives, AxioCam MrM CCD camera. Axiovision software was used for image acquisition (Carl Zeiss).

RNA interference
Hypoxia-inducible factor-1α, HIF-2α, PHD2 and Notch1 siRNA oligonucleotides were obtained commercially (Santa Cruz Biotechnology). A previously designed siRNA directed against green fluorescent protein was used as a control. The day before transfection, 5×10^6 CSCs were dissociated into single cells and transferred to T25 Falcon culture flasks with suspension medium containing EGF. Cells were then transfected with siRNA using a nucleofecting electroporator according to the manufacturer's protocol (Amaxa Inc., Gaithersburg, MD, USA). After 24 h, the medium was replaced, and cells were harvested for additional experiments.

Statistical analysis
Differences in the various surface maker expressions between normoxia and hypoxia were evaluated with Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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革新的癌治療薬となる遺伝子・ウイルス治療薬と肝疾患への再生医薬

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1．はじめに

遺伝子治療と再生医療は，21世紀の革新的治療法としてその開発が期待されている．筆者らは，種々の難治性疾患に対して両治療法の開発を進めてきたが，本稿では，特に実用化を具体的に目指している独自開発の多因子制御による癌特異的増殖型アデノウイルスベクター(m-CRA；conditionally replicating adenovirus regulated with multiple tumor-specific factors)による癌治療，増殖因子のHB-EGF(Heparin-binding epidermal growth factor-like growth factor)による肝疾患への再生医薬について，その現状と展望を紹介する．

2．癌に対する遺伝子・ウイルス治療薬

2．1　癌遺伝子治療の背景と問題点

癌は，本邦や先進諸国における三大死因で年間死亡数も依然増加しており，革新的な癌治療法の開発は本邦の医療福祉の向上の観点からも最重要課題である．その有力候補の一つの遺伝子治療は，1991年の最初の癌患者への臨床試験以来，現在まで癌だけでも960の臨床プロトコールが世界中で認可され，多くの癌患者へ臨床試験がなされている．その結果，安全性はよく確認されたが，治療効果が当初期待されたような癌根治に近づくレベルには達していないことが明らかとなった．全ての癌治療法の根幹の問題は，“全て”の癌細胞を殺してしまわなければ再発して根治には至らないということであり，遺伝子治療の成功の鍵はそれを可能とする画期的な遺伝子導入ベクターと治療遺伝子を開発することに他ならない．

第一世代の癌遺伝子治療は，1990年代に臨床試験が進められたレトロウイルスによるサイトカイン遺伝子の*ex vivo*導入法(切除癌細胞に*in vitro*培養下で遺伝子導入し，放射線で増殖不能化して体内に戻す)による癌免疫ワクチン療法である．これは，治療効果が不十分なことに加えて，多大な経費や労力の点からも一般医薬化には至らなかった．第二世代の癌遺伝子治療は，1990年代前半に開発された*in vivo*遺伝子治療(癌結節に直接ベクターを注射して遺伝子導入する)であり，その主たるベクターはアデノウイルス(ADV；adenovirus)ベクターである．これでも治療効果が不十分だった主因の一つは，これらのベクターは“非”増殖型のベクターであるため，つまり*in vitro*でいくら遺伝子導入効率の高いベクターでも，*in vivo*の体内の全ての癌細胞にもれなく遺伝子を導入することは物理的に不可能，つまりベクター液の未到達の遺伝子“未”導入癌細胞の再増大，再発が起こりえるからである(図1)．筆者らが米国で開発した全身性抗腫瘍免疫を効率よく獲得する“自殺遺伝子/サイトカイン遺伝子のコンビネーション遺伝子治療”[1,2]などいくつかの戦略は，遺伝子“未”導入癌細胞もある程度治療できる可能性はあるものの，その根本解決として現在開発が進められているのは癌特異的な増殖型ウイルスによる第三世代の遺伝子治療である．癌特異的増殖型ウイルスの代表はADVをベースとしたもので，CRA(Conditionally replicating

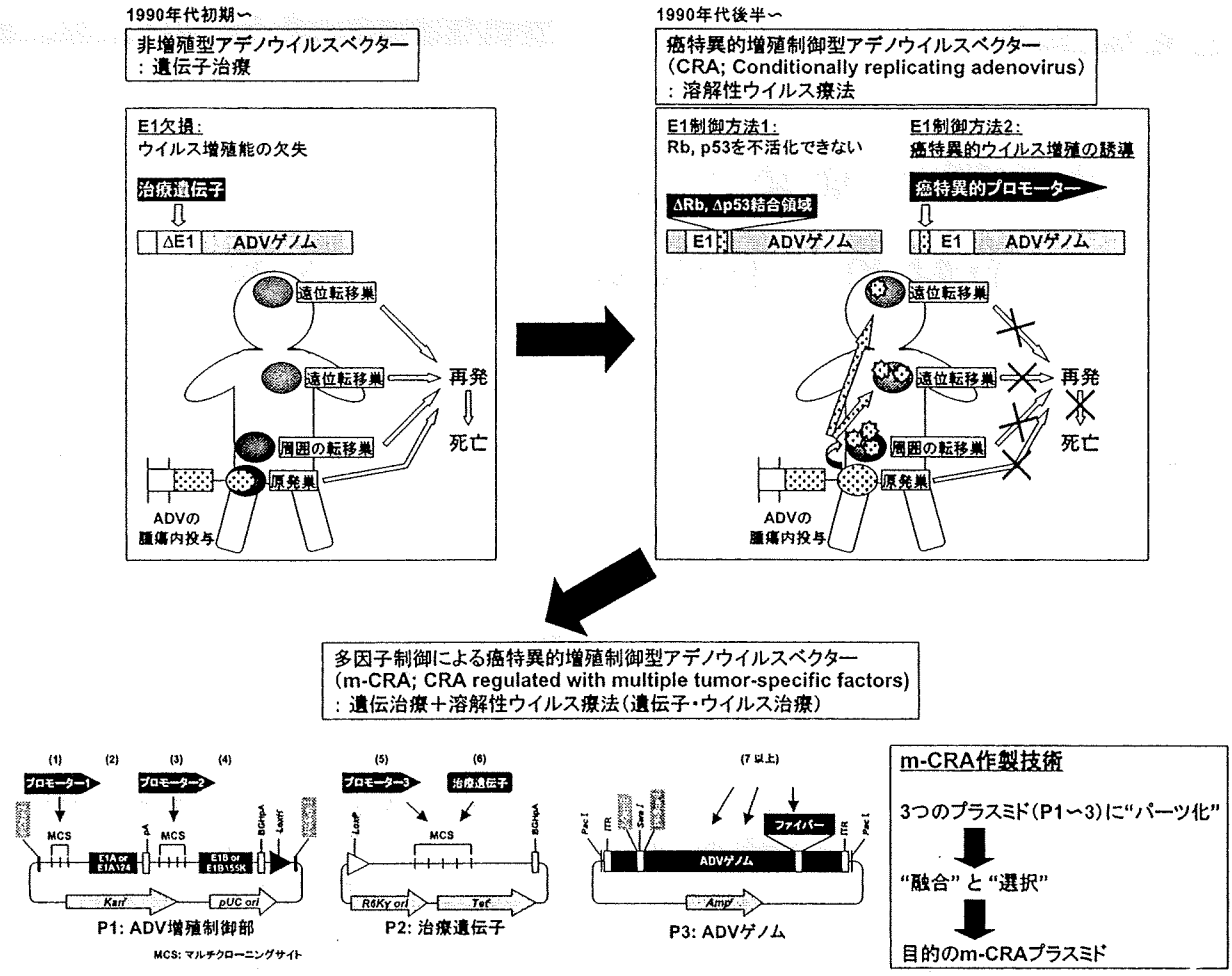

図1　癌遺伝子治療法の変遷と m-CRA 作製技術の開発

adenovirus)あるいは溶解性アデノウイルス(Oncolytic adenovirus)と呼ばれる(図1)．CRA は，遺伝子治療で培われたウイルスベクター技術を基盤としている点から遺伝子治療分野の一つではあるが，溶解性ウイルスという別名のようにその癌細胞殺傷機序は癌細胞内で増幅したウイルス蛋白質である．また，実際これまでの CRA は外来性の治療遺伝子を搭載していないタイプが主体であることからも，癌治療法という観点からみた場合はウイルス治療(Virotherapy)という名前が理解しやすいと思われる．いくつかの CRA は臨床試験が進められ，ある程度の安全性は確認されているものの，以下に述べる技術的制約のためいくつかの課題が残されていた．

2．2　m-CRA 作製技術の開発

ADV の増殖に必須の領域は E1(いくつかの蛋白コード遺伝子を含む)であるため，第二世代癌遺伝子治療の非増殖型 ADV ベクターはこの E1 領域を欠損させて，治療遺伝子で置換するというものであった．一方，CRA は癌特異的なウイルス増殖を誘導するためにこの E1 を制御するものであり，その方法は大きく二つに分けられる(図1)．一つは，ADV の E1 領域内の Rb，p53 結合領域を欠損させる方法である．つまり，この変異 ADV である CRA は，ウイルス増殖に必要な“細胞周期が回っている”という細胞環境の誘導に必須となる“Rb や p53 の不活化”を行えなくなるため，正常細胞でのウイルス増殖は阻止されることになる．一方で，細胞周期が制御なしに異常に回っている癌細胞はこれに関係なくこの CRA も増殖し，癌細胞を殺傷する．もう一つは，E1 遺伝子の内因性プロモーターを癌特異的に発現する遺伝子のプロモーターに置換することで，癌特異的ウイルス増殖を誘導するものである．いずれもこれまでの臨床試験で大きな有害事象は報

告されておらず，優れた戦略ではある一方で，たかだか1因子で癌と正常の細胞を完全に区別することは難しいであろうという癌特異化(潜在的な安全性)の問題，ウイルス蛋白質のみに依存した治療効果の問題などが大きな課題として残っていた．そして，その課題を解決できない根幹の原因は非増殖型ADVとは異なり，CRAは未だ効率的な標準化作製技術がないということである．つまり，そのため研究は非効率で，またより複雑に改変された高度なCRAの開発も困難だったのである．

これを解決するために筆者らは，上記のE1に関する四つの癌特異化因子(変異E1A，変異E1Bをそれぞれ異なる癌特異的プロモーターで発現制御する)で同時に精密にウイルス増殖を制御すること(安全性の向上)が可能で，さらに治療遺伝子を搭載(癌治療効果を向上)し，ADVゲノム部(感染指向性を決めるファイバー部などウイルスの性質決定部)の変更も簡単にできる，第四世代の遺伝子治療となるm-CRA(CRA regulated with multiple tumor-specific factors)作製技術を開発した(図1)[3]．また，これは第二世代の“遺伝子治療”と第三世代の“ウイルス療法”の長所を融合した“遺伝子・ウイルス治療(Gene-Viro-Therapy)”となる，革新的な癌治療法へと展開できる可能性を持つ．m-CRA作製技術の原理，プロトコールの詳細はすでに前著にて報告しているので[3,4]，本稿では要点だけを示す．その発想は，まずm-CRAの構成要素を“パーツ化”して，ADV増殖制御部E1，治療遺伝子，ADVゲノムを異なる三つのプラスミドに収載することで，各部分を独立して簡単に遺伝子組換えすることを可能とした．その後は，三つのプラスミドを簡単に“融合”させて，正しいものだけを確実に“選択”して一つのm-CRAプラスミドまで作製できるように種々の工夫を施している．

従来の“手作り”でのCRAの作製効率(例えば専門家が年に1，2個作製)と比較すれば，筆者らの“システム・プロトコール”化されたm-CRA作製技術は小スケール調整で同時に多種多様な検体を扱えることより，多検体の作製における作業効率は桁違いの向上率となるものである(例えば，テクニシャンが年間に数十個作製など)．しかも，医薬あるいはGene delivery toolとしての性能が従来のCRAを大きく凌ぐm-CRAも作製することができる．また各部をパーツ化しているため，バンク化したパーツの組み合わせでさらに多種多様のm-CRAの作製が可能で，またm-CRAの一部の改変も極めて容易である．このm-CRA作製技術により，CRA，m-CRAの受託作製の事業化も初めて可能となるため，筆者らはベンチャー創業(平成22年3月予定)により自身らで癌への遺伝子・ウイルス治療薬の創薬・臨床開発を進める一方で，優れたGene delivery toolあるいは癌治療研究用として研究者や企業へ受託作製の事業化を行う計画をし，その準備を進めている．

2．3　癌に対する遺伝子・ウイルス治療医薬としてのm-CRA

筆者らがm-CRA作製技術により，当初種々のm-CRAを作製して解析したが，医薬化を進める目的でオリジナル癌治療医薬として開発した第一弾m-CRAはSurvivin反応性m-CRA(Surv.m-CRA)である[5]．Survivinは，1997年にアポトーシス阻害蛋白質(IAP)ファミリーの一つとして同定されたが，その後ほとんどの癌で高発現し，分化した正常細胞ではほとんど発現していないことが分かった．さらにSurvivinの発現が癌の悪性度と相関することも明確となり，現在Survivin抑制を目指した医薬開発も試みられている．筆者らはまず，Survivinプロモーターで E1A(あるいは変異E1A)を発現制御し，E1Bも変異化した2，3因子制御のSurv.m-CRAを開発した．Surv.m-CRAは，*in vitro* および *in vivo* において非常に高い癌特異性を示し，さらに既存の中では最も優れたCRAの一つと思われるテロメラーゼ(hTERT；human telomerase reverse transcriptase)反応性m-CRA(Tert.m-CRA)との詳細な比較実験でも，Surv.m-CRAはTert.m-CRAよりも癌特異性(安全性)および癌治療効果の両面で遥かに凌ぐ性能を示した[5]．上記のようにSurvivinはほとんどの癌で高発現していることが知られているため，Surv.m-CRAは多くの癌を対象とした画期的な癌治療薬となることが期待される．

さらに筆者らは，癌治療効果と癌特異化(安全

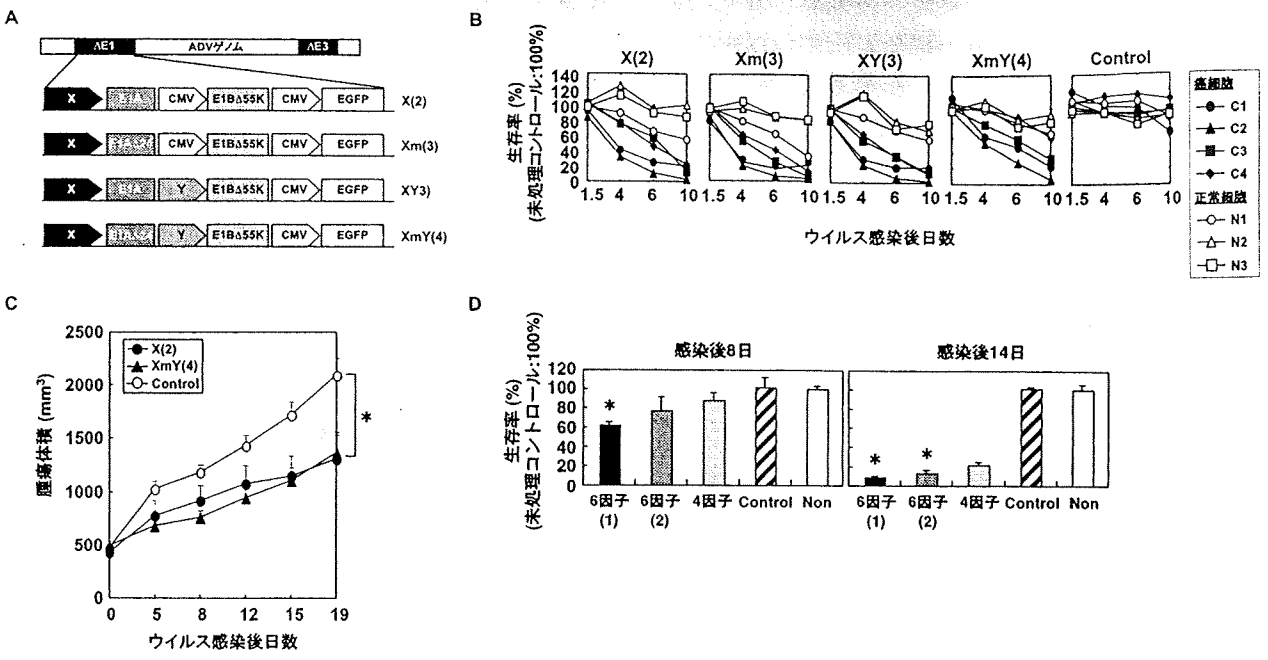

図2　癌に対する遺伝子・ウイルス治療医薬としてのm-CRA

性)がさらに向上した新型Surv.m-CRAを開発する目的ならびに最適のm-CRA化の一般法則とそのメカニズムを同定する目的で，種々のSurv.m-CRAを含むm-CRAを種々作製し，解析している．(新型Surv.m-CRAは未だ発表できないため)，ここでは2種類の異なる癌特異的遺伝子のプロモーター(X，Y)，E1AとE1Bの野生型/変異型を組み合わせた最大4因子の異なる癌特異化因子で増殖制御する4種類のm-CRAの解析結果を示す(図2A～C)．比較解析の結果，*in vitro*において4種類の全てのm-CRAは癌特異的ウイルス増殖と強い癌治療効果を示した(図2B)．また，より多くの癌特異化因子(最大の4因子)で増殖制御することにより，癌治療効果を維持したまま正常細胞での細胞傷害を抑制する，つまり癌特異性を向上できるという成果が得られた．さらには*in vivo*においても同様の結果が得られ，4因子制御m-CRAは*in vitro*において最も強い癌治療効果を示した2因子制御m-CRAと同等に腫瘍の増殖抑制・退縮を示した(図2C)．つまり，E1をより高度にm-CRA化することで“癌治療効果を減弱することなく”，さらに“癌特異性(安全性)を向上することが可能である”ことが明確となった(図2A～C)．筆者らはさらに，このE1を4因子制御したより癌特異性(安全性)が増したm-CRAに，さらに2種類の異なる治療遺伝子ユニット(癌特異的治療遺伝子を癌特異的プロモーターで発現制御する)を組込んだ6因子制御m-CRAを作製し，比較実験をしてさらに治療効果を増強するm-CRAを開発することにも成功している(図2D)．また，癌への感染指向性が向上したファイバー改変型m-CRAも作製，解析しており，これらの成果を導入した7因子制御の新型Surv.m-CRAを現在開発しているところである．上記のように(2，3因子制御の筆者らにとってプロトタイプの)，Surv.m-CRAですら従来のCRAの性能を凌いでいたため，従来のCRAの課題を克服しつつある新型Surv.m-CRAは画期的な癌治療医薬となるものと思われる．

また，筆者らはこれまでに標的とされていない癌特異化因子あるいは分子機構を標的とした，Surv.m-CRAに続く第二，第三弾のm-CRA開発も進めている．一方，上記のようにm-CRAベクターは画期的なGene delivery toolにもなるため，医薬開発あるいは研究用として有用なshRNA発現m-CRAシステムも開発を進めている．これらのシステムも順次，医薬開発あるいは研究や開発支援の受託作製の事業へと展開して行きたいと思っている．

3．肝疾患に対する再生医薬としてのHB-EGF

3．1　再生医学の背景

再生医学(医療)とは，失われた組織を補完するさまざまな治療法の総称であるが，以下の二つに大別することができる[6]．一つは昨今流行のiPS細胞やES細胞といった多能性幹細胞により目的細胞を分化誘導・単離して細胞移植療法に用いるというもので，臓器移植に代わる治療法を目指した，“再建医学”ともいうべきものである．もう一つの再生医学は，何か物質を投与したらトカゲの尻尾のように失われた臓器が蘇ってまさに“再生”してくるというような，おそらく一般の人が思い描いている再生医学に近いものである．“生体内再生医学”ともいうべき後者の適応臓器は，生後も再生能を保持している肝臓，皮膚，血管，血球などに限られるため，筆者らも心臓や神経などの臓器疾患には前者の“再建医学”を主体として，ES細胞などで独自の技術開発を進めている[6~8]．一方，肝臓はヒトでも生体肝移植が可能なようにその再生能力が極めて高いことから，筆者らは肝臓はまさに“生体内再生医学(医薬)”の理想的な対象臓器と考え，その開発を行ってきた．特に本邦やアジア諸国では肝疾患者数は多く，急性肝炎は本邦だけで毎年約20万人の発症があるといわれているように，肝疾患への画期的な治療薬の開発は重要である．多くの肝疾患，例えば急性肝炎への既存の医薬，医療は単なる対症療法にすぎず，極論すれば安静，栄養補給だけで肝臓の元来の再生能力に全面的に依存して自然治癒を待っているだけであり，つまり“病気の進展をほぼ完全に止め，治癒再生を劇的に促す”根治医薬というものは未だ存在しないのが現状である．移植以外に有効な治療法がない，短期間で極めて高い致死率を示す(数日から数週間で60～70%の致死率)という点で，劇症肝炎は代表的な難治性肝疾患である．そこで筆者らは初めに，劇症肝炎に対する生体内再生治療法(薬)の開発を，増殖因子のHGF(Hepatocyte growth factor)を用いて行った[9~11]．

HGFは，肝再生の本体の因子として1984年に単離，1989年にクローニングされた増殖因子である．血清HGFは劇症肝炎患者で急激な上昇がみられ，予後判定のマーカーともなっているが，血清で上昇しているHGFは一本鎖や断片化した活性を持たないあるいはむしろ活性を阻害しているHGFも多分に検出されていることも示唆されている．よって，活性型の二本鎖の組換えHGF蛋白質を多量投与して人為的に補うことで，本来の生理的効果を回復し，劇症肝炎の発症が阻止，治療できるのではないかと考え，二つの劇症肝炎モデルで治療実験を行った[10,11]．その結果，当時分かっていたHGFの強力な肝再生誘導作用だけでなく，このHGFの抗アポトーシス効果を中心とする肝細胞死の抑制作用が極めて強力であった[10~12]．つまりヒト劇症肝炎は，数日から数週間で60～70%が死亡するものであるが，Fas誘導，エンドトキシン誘導の二つの劇症肝炎マウスモデルは，これとは比較にならないほど急激かつ重篤な劇症肝炎モデルである．特に後者は，肝障害発症から3～4時間以内に100%死亡するという激烈な劇症肝炎マウスモデルであるが，HGF投与によりその死亡率は30%以下へと強力に抑制された[10,11]．このように強力な治療効果と，そして元来の生体内物質であるという点からも，科学的結果からはHGFの蛋白質製剤は急性/劇症肝炎への根治薬となりえるものと推察される．

3．2　HB-EGFによる肝再生医薬の開発と展望

筆者らは，このようにHGFをモデルとして，“病気の進展(細胞死)を止め，同時に障害臓器を再生治癒する”という理想的な生体内再生医薬の戦略を初めて報告した．しかし，一方でHGF自体はすでにさまざまな分野で研究が進み，また，医薬開発も発見者を中心に種々進められている．そこで筆者らは，自身で提唱した理想的な“生体内肝再生医薬”の戦略は基盤としながらも，医薬応用はまだなされておらず，しかもHGFを凌ぐ性能を持つ可能性のある物質としてHB-EGF(Heparin-binding epidermal growth factor-like growth factor)を候補と考えその検証を行った．

HB-EGFは，最初はジフテリアトキシン受容体として発見されたが，その後EGFファミリーに属する新規増殖因子としての役割を併せ持つことが明らかとなった．HB-EGFはインテグリン

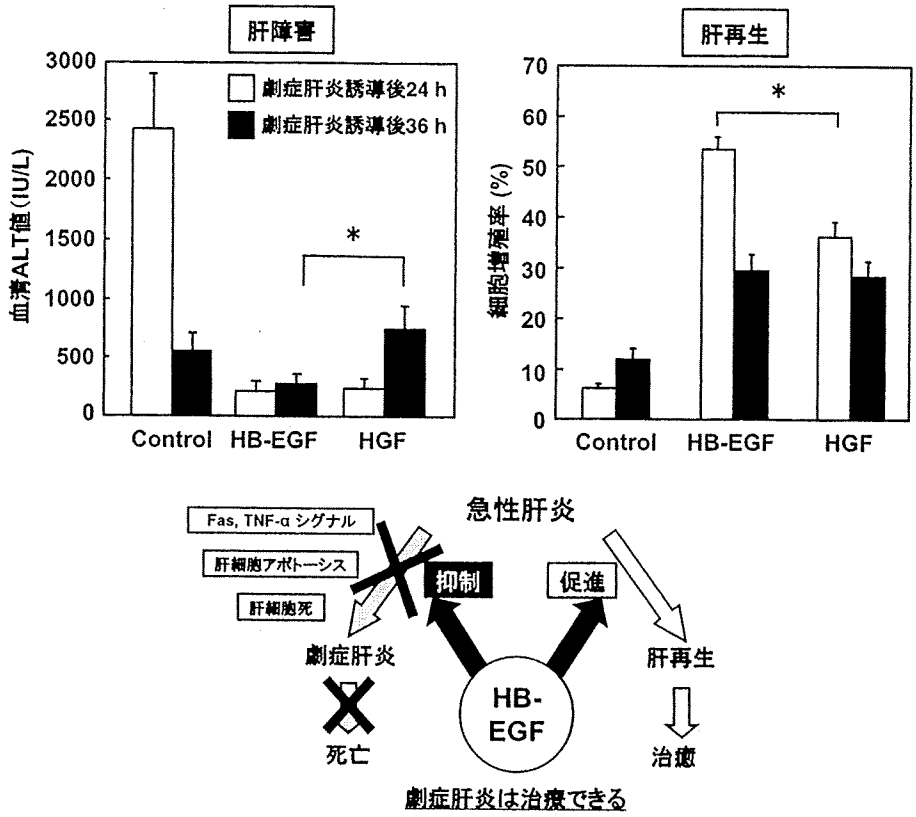

図3　肝疾患に対する再生医薬としてのHB-EGF

やTetraspaninファミリーなどとアソシエートする膜結合型増殖因子であり，まずは膜アンカー型のproHB-EGFとして産生された後に，特定のメタロプロテアーゼにより切り出されて，活性型の可溶性HB-EGFが産生される．肝障害や肝再生の際にはクッパー細胞や類洞内皮細胞でHB-EGFの発現が上昇し，特に興味深いのは肝障害後にはHGFよりも早期に急激にHB-EGFは上昇するということである．しかし，治療への応用という観点からさまざまな臓器疾患で研究が進められているHGFとは異なり，それまでHB-EGFの医薬応用の可能性について検証した報告はなかった．特に，生物学レベルでは多少想像された肝再生誘導作用とは違い，抗アポトーシス作用あるいは肝細胞死の阻止作用という観点でのHB-EGFの研究は報告がなかった．

筆者らは，遺伝子治療の専門家であり，上記のようなADVベクター技術を持つためHB-EGFならびにHGFを発現するADVベクターをそれぞれ作製し，マウスの尾静脈投与による肝細胞へのHB-EGF，HGF遺伝子導入・強発現によりFas誘導の急性肝炎に対する肝障害抑制作用，肝再生誘導作用を比較した(図3)[13]．その結果，HB-EGFはHGFよりもある時点で強力な肝障害抑制効果を示し，さらに肝障害早期の肝再生誘導効果はHB-EGFはHGFを大きく凌いでいた．つまり，上記のように強力な肝細胞死の抑制効果と肝再生誘導効果を示したHGFの両作用をさらに凌ぐHB-EGF医薬の性能は，医薬として極めて高く期待できるものと結論づけられる(図3)．今回は遺伝子治療(導入)の実験手技を用いたが，実際に第一適応として開発を進める急性/劇症肝炎への医薬形態としては，遺伝子医薬より蛋白質製剤の方がより現実的で有用だと思い，そのように医薬開発を進めて行きたいと思っている．

HB-EGF生体内再生医薬の長所をまとめると，以下のようになる．①肝疾患への既存医薬は対症療法にすぎないが，HB-EGFは根治薬となる可能性が大きい．②投与法は簡便である(肝障害をみつけたらHB-EGFの静脈注射で病気は止まり，治癒に向かう)．③HB-EGFはHGFよりさらに肝細胞死抑制，肝再生誘導の両効果とも強い(特に急性肝障害への再生誘導効果は際立っている)．④応用特許としても新規性が高い(特許性高い；

まだHGFほど研究されてない)．⑤急性肝炎への蛋白質製剤としての開発は極めて現実的である．⑥活性を持つ組換え蛋白質を大腸菌で産生可能．よって，GMPでの大量生産も容易と思われる．⑦他の肝疾患へも適応拡大が期待できる．このようにHB-EGFの再生医薬としての実用化の可能性は大きく，肝硬変をはじめ他の肝疾患への治療効果の検証を進める一方で，一刻も早い一般製薬化実現による患者さんへの恩恵のため，急性/劇症肝炎へのHB-EGF蛋白質製剤の臨床開発をいずれかの製薬会社と共同で進めて行ければと願っている．

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Treatment with an adenoviral vector encoding hepatocyte growth factor mitigates established cardiac dysfunction in doxorubicin-induced cardiomyopathy

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[1]*Division of Cardiology, Gifu University Graduate School of Medicine, Gifu;* [2]*Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, Kurume; and* [3]*Department of Food Science, Kyoto Women's University, Kyoto, Japan*

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Esaki M, Takemura G, Kosai K-i, Takahashi T, Miyata S, Li L, Goto K, Maruyama R, Okada H, Kanamori H, Ogino A, Ushikoshi H, Minatoguchi S, Fujiwara T, Fujiwara H. Treatment with an adenoviral vector encoding hepatocyte growth factor mitigates established cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Am J Physiol Heart Circ Physiol* 294: H1048–H1057, 2008. First published December 14, 2007; doi:10.1152/ajpheart.01102.2007.— Hepatocyte growth factor (HGF) reportedly exerts beneficial effects on the heart following myocardial infarction and during nonischemic cardiomyopathy, but the precise mechanisms underlying the latter have not been well elucidated. We generated nonischemic cardiomyopathy in mice by injecting them with doxorubicin (15 mg/kg ip). Two weeks later, when cardiac dysfunction was apparent, an adenoviral vector encoding human HGF gene (Ad.CAG-HGF, 1×10^{11} particles/mouse) was injected into the hindlimb muscles; LacZ gene served as the control. Left ventricular dilatation and dysfunction normally seen 4 wk after doxorubicin administration were significantly mitigated in HGF-treated mice, as were the associated cardiomyocyte atrophy/degeneration and myocardial fibrosis. Myocardial expression of GATA-4 and a sarcomeric protein, myosin heavy chain, was downregulated by doxorubicin, but the expression of both was restored by HGF treatment. The protective effect of HGF against doxorubicin-induced cardiomyocyte atrophy was confirmed in an in vitro experiment, which also showed that neither cardiomyocyte apoptosis nor proliferation plays significant roles in the present model. Upregulation of c-Met/HGF receptor was noted in HGF-treated hearts. Among the mediators downstream of c-Met, the activation of extracellular signal-regulated kinase (ERK) was reduced by doxorubicin, but the activity was restored by HGF. Levels of transforming growth factor-β1 and cyclooxygenase-2 did not differ between the groups. Our findings suggest the HGF gene delivery exerts therapeutic antiatrophic/degenerative and antifibrotic effects on myocardium in cases of established cardiac dysfunction caused by doxorubicin. These beneficial effects appear to be related to HGF-induced ERK activation and upregulation of c-Met, GATA-4, and sarcomeric proteins.

heart failure

THE ANTINEOPLASTIC DRUG doxorubicin is highly effective against a broad spectrum of hematogenous and solid human malignancies, but its clinical use is limited by its adverse side effects: irreversible degenerative cardiomyopathy and congestive heart failure (29, 32). Much effort has gone into the search for treatments able to reduce or eliminate the risk of doxorubicin-induced cardiomyopathy and congestive heart failure (11, 31, 33), but so far the ability of these treatments to protect the heart from damage has been varied and limited.

Hepatocyte growth factor (HGF), which was originally identified and cloned as a potent mitogen for hepatocytes (25, 26), has been shown to exert mitogenic, angiogenic, antiapoptotic, and antifibrotic effects in various cell types, especially in epithelial and endothelial cells (5, 14). Moreover, HGF also reportedly exhibits cardioprotective effects. For instance, HGF protected cardiomyocytes from acute ischemic death during myocardial infarction (27, 36), and it enhanced survival among cardiomyocytes subjected to oxidative stress (13, 36). In addition to its beneficial effects on cardiomyocytes under acute stress, recent research has demonstrated that HGF also exerts beneficial effects on cardiac function in animal models of chronic heart diseases, including ischemic cardiomyopathy following old myocardial infarction and hereditary cardiomyopathy (18, 28, 34). In those cases, the main mechanisms appeared to be a hypertrophic effect on cardiomyocytes as well as angiogenic and antifibrotic actions. More recently, Iwasaki et al. (12) reported that HGF prevents cardiac dysfunction in an animal model of doxorubicin-induced cardiomyopathy. In that study, however, HGF was administered as the protein form, and the delivering method of HGF was very specific (intravenous injection of HGF delivered by ultrasound-mediated destruction of microbubbles). The effect of the HGF gene therapy has not been reported so far on established cardiac dysfunction due to doxorubicin cardiomyopathy.

Our hypothesis in the present study was that late treatment with HGF gene transfer can mitigate established heart failure brought on by doxorubicin-induced cardiomyopathy. To test that idea, we used an adenoviral vector to transfer the human (h)HGF gene into mouse hindlimbs (systemic transfection) 2 wk after doxorubicin injection, a time at which cardiac dysfunction was already apparent. We then examined the effects of the gene on the progression of heart failure during a more chronic stage and investigated the specific mechanisms involved.

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MATERIALS AND METHODS

Recombinant adenoviral vectors. The adenoviral vector plasmid pAd-HGF, which is comprised of the cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter, rabbit β-globin polyA (CAG) and hHGF cDNA (Ad.CAG-HGF), was constructed using the in vitro ligation method described previously (18, 21). Control Ad-LacZ (Ad.CAG-LacZ) was prepared as described previously (7).

Measurement of hHGF levels. hHGF levels in plasma and tissues (n = 3 to 4/group) were measured using an ELISA kit (Institute of Immunology) as previously reported (18).

Experimental protocols. This study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. Cardiomyopathy was induced in 10-wk-old male C57BL/6J mice (Chubu Kagaku, Nagoya, Japan) by a single intraperitoneal injection of doxorubicin (doxorubicin hydrochloride, Kyowa Hakko, Tokyo, Japan) at a dose of 15 mg/kg. We previously confirmed both functionally and histologically that, at that dosage, doxorubicin induces cardiomyopathy in all mice not receiving a therapeutic intervention (16). In sham-operated mice, the same volume of saline was injected in a similar manner.

In the first set of experiments (*protocol 1*), treatment with adenoviral vectors was started 2 wk after saline or doxorubicin injection; Ad.CAG-HGF or Ad.CMV-LacZ at 1×10^{11} particles/mouse was injected into the hindlimb muscles of 10-wk-old male C57BL/6J mice. At that time, mice were assigned to receive saline alone (n = 11), doxorubicin plus LacZ gene (n = 10), or doxorubicin plus hHGF gene (n = 9). Before this assignment, echocardiography was done to reduce any bias among the groups. After an additional 2 wk (4 wk after doxorubicin administration), all mice received a physiological examination and were then euthanized with an overdose of pentobarbital sodium. Cardiac specimens were then collected and subjected to histological, immunohistochemical, and molecular biological analyses.

In a second set of experiments (*protocol 2*), we evaluated the role played by the extracellular signal-regulated protein kinase (ERK) signaling pathway in mediating the effects of the hHGF gene therapy. PD-98059 (Cell Signaling), a MEK1-p42/p44 mitogen-activated protein kinase (MAPK)-specific inhibitor (8), was administered intraperitoneally at a dose of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 wk to mice given saline or doxorubicin plus hHGF (n = 7 each), after which the mice were examined as described in *protocol 1*.

In vitro study. Cardiomyocytes were isolated from 1-day-old neonatal C57BL/6J mice as previously reported (3) and plated on laminin-coated dishes or in slide glass chambers and incubated in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS (Sigma) for 48 h at 37°C. Doxorubicin was then added to the medium to a final concentration of 0.1 μmol/l. Simultaneously, recombinant hHGF (Wako) was added to a concentration of 0, 0.02, 0.2, 2, or 10 ng/ml. For the controls, doxorubicin and/or HGF was replaced with the same volume of saline. Twenty-four hours later, the cells were collected for morphometric and biochemical analyses.

The cardiomyocytes in slide glass chambers were fixed in 4% paraformaldehyde, permeabilized with 0.05% Triton X, and stained with rhodamine phalloidin and Hoechst 33342 (both from Molecular Probes). Digital images captured using a laser-confocal microscope system (LSM510, Zeiss) were employed for morphometric analysis using Photoshop 7.0 (Adobe Systems). Proteins extracted from cardiomyocytes on dishes were used for Western blot analysis.

Physiological studies. Echocardiography and cardiac catheterization were carried out as described previously with modifications (16). Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N_2O and O_2 (0.5 l/min each) via a nasal mask. Echocardiograms were recorded using an echocardiographic system (Vevo770, Visualsonics) equipped with a 45-MHz imaging transducer before treatment and at death. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) that was advanced into the aorta and then into the left ventricle for recording pressure and maximal and minimal first derivative of pressure ($\pm dP/dt$).

Histological analysis. Following echocardiography, each heart was removed and cut into three transverse slices. Of those, the middle slice was fixed with 10% buffered formalin and embedded in paraffin, after which 4-μm-thick sections were stained with hematoxylin-eosin or Sirius red F3BA (0.1% solution in saturated aqueous picric acid) (Aldrich). Quantitative assessments, including cell size and cell number, were carried out in randomly chosen high-power fields (HPFs) in each section using a multipurpose color image processor (LUZEX F, Nireco). The fibrotic area was measured by searching the entire ventricle. Cardiomyocyte size (expressed as the transverse diameter of myocytes cut at the level of the nucleus) and cell populations were assessed in 20 randomly chosen HPFs in each section.

Immunohistochemistry. After deparaffinization, the 4-μm-thick sections were incubated with a primary antibody against c-Met/HGF receptor (Santa Cruz), endothelial cells (Flk-1, Santa Cruz), leuko-

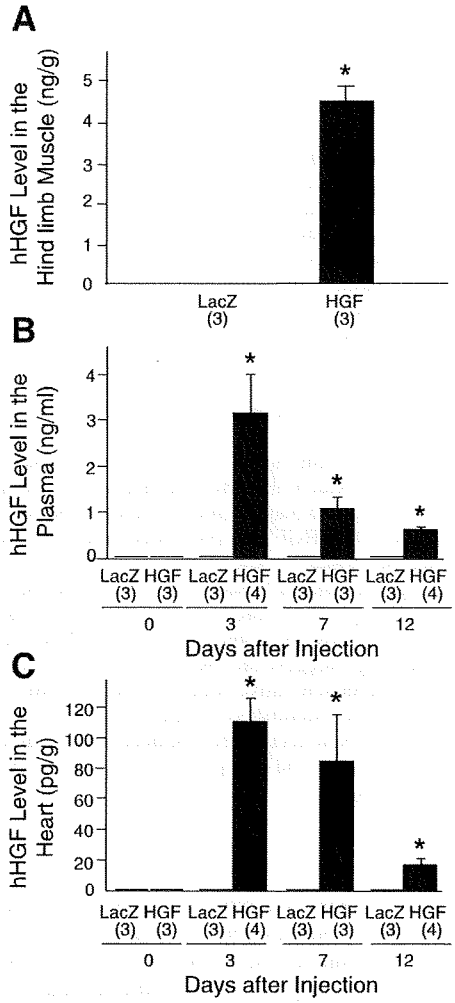

Fig. 1. *A*: human hepatocyte growth factor (hHGF) levels in the hindlimb muscles of mice injected with adenoviral vector 3 days earlier were detected by ELISA. *B*: time course of hHGF levels in the plasma of mice after adenovirus injection. *C*: time course of hHGF levels in the heart. Numbers in parentheses indicate those of animals used per group. *$P < 0.05$ vs. doxorubicin (Dox) + LacZ group.

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cytes (CD45, Pharmingen), or Ki-67 (Santa Cruz). A Vectastain Elite ABC system (Vector) was then used to immunostain the sections; diaminobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin. Quantitative assessments, including the number or area of the immunopositive cells, were made in 20 randomly chosen HPFs using the multipurpose color image processor.

In situ terminal dUTP nick-end labeling (TUNEL) assays were carried out with sections using an ApopTag kit (Chemicon) according to the supplier's instructions. Mouse mammary tissue served as a positive control.

Electron microscopy. Cardiac specimens were immersion fixed overnight in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), post-fixed for 1 h with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in an electron microscope (H700, Hitachi, Tokyo, Japan).

Western blot analysis. Heart tissue lysates were used for Western blot analysis. Proteins were separated and transferred to membranes using standard protocols, after which they were probed using antibodies against GATA-4 and myosin heavy chain (MHC) (both from Santa

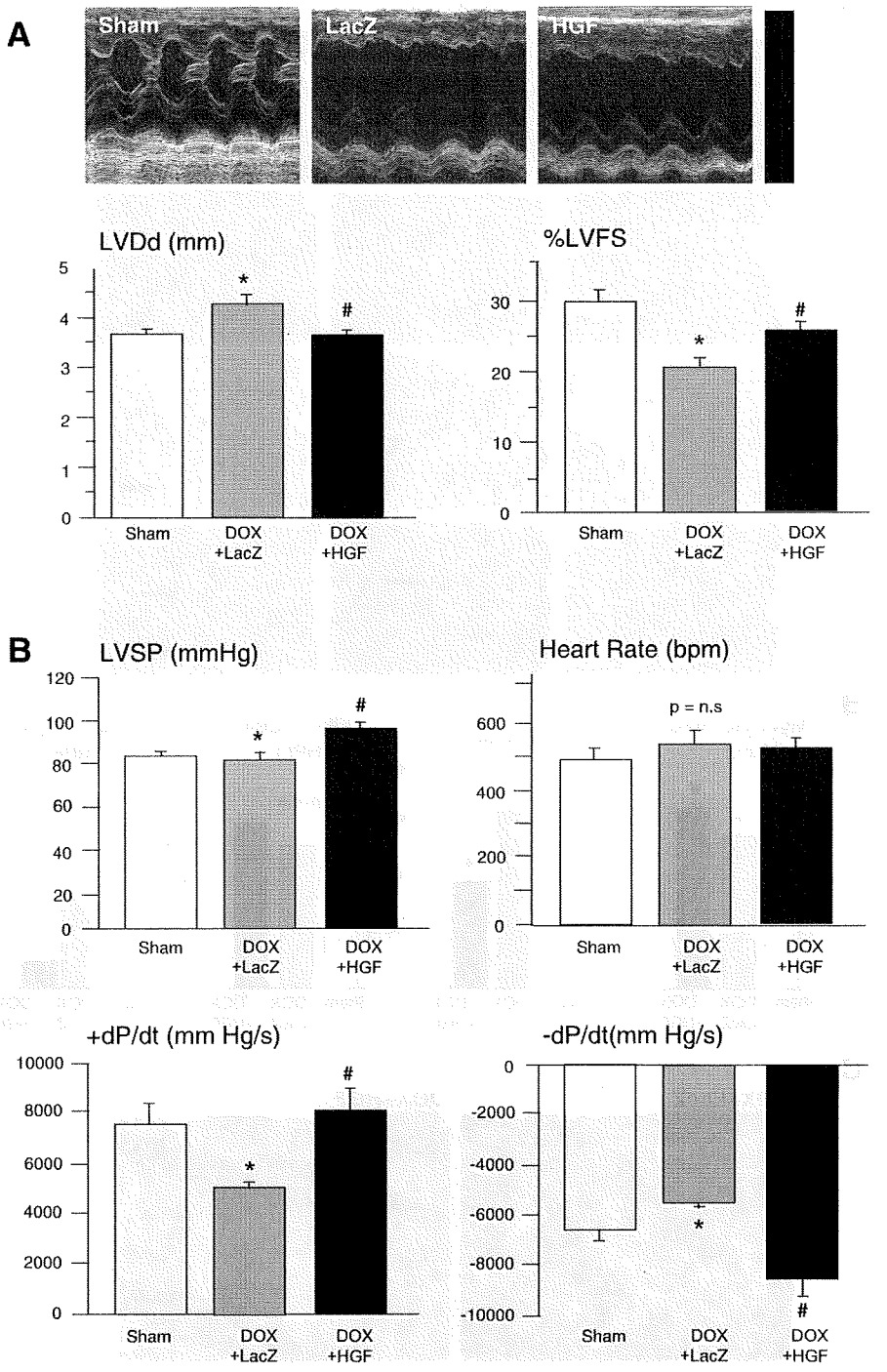

Fig. 2. Effects of hHGF gene delivery on cardiac remodeling and function assessed 4 wk after Dox injection (*protocol 1*). The indicated parameters were measured using echocardiography (*A*) and cardiac catheterization (*B*). LVDd, left ventricular (LV) end-diastolic diameter; %LVFS, %LV fractional shortening; LVSP, LV peak systolic pressure; bpm, beats/min; ±dP/d*t*, maximum and minimum first derivative of pressure. $*P < 0.05$ vs. sham group; $\#P < 0.05$ vs. Dox + LacZ group.

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Cruz), transforming growth factor-β1 (TGF-β1, Promega), cyclooxygenase-2 (Santa Cruz), and Akt or ERK (both from Cell Signaling). Activation of Akt and ERK was assessed using antibodies against the phosphorylated form of Akt (p-Akt) and p-ERK (both from Cell Signaling), respectively. Procaspase-3 and the activated form of caspase-3 were evaluated using anti-caspase-3 antibody (Santa Cruz). Western blot analysis of hHGF was performed using anti-human IgG antibody (DAKO). Three to five hearts from each group were subjected to the blotting. The blots were visualized by means of enhanced chemiluminescence (Amersham), and the signals were quantified by densitometry. α-Tubulin (analyzed using an antibody from Santa Cruz) served as the loading control.

Immunoprecipitation and Western blot analysis for c-Met. Heart tissue lysates were subjected to immunoprecipitation assays carried out with Ultra-Link Biosupport medium (Pierce) using anti-c-Met antibody (Santa Cruz). Thereafter, the immunoprecipitate was analyzed by Western blot analysis using the same antibody. Three to five hearts from each group and three normal livers were subjected to the assay.

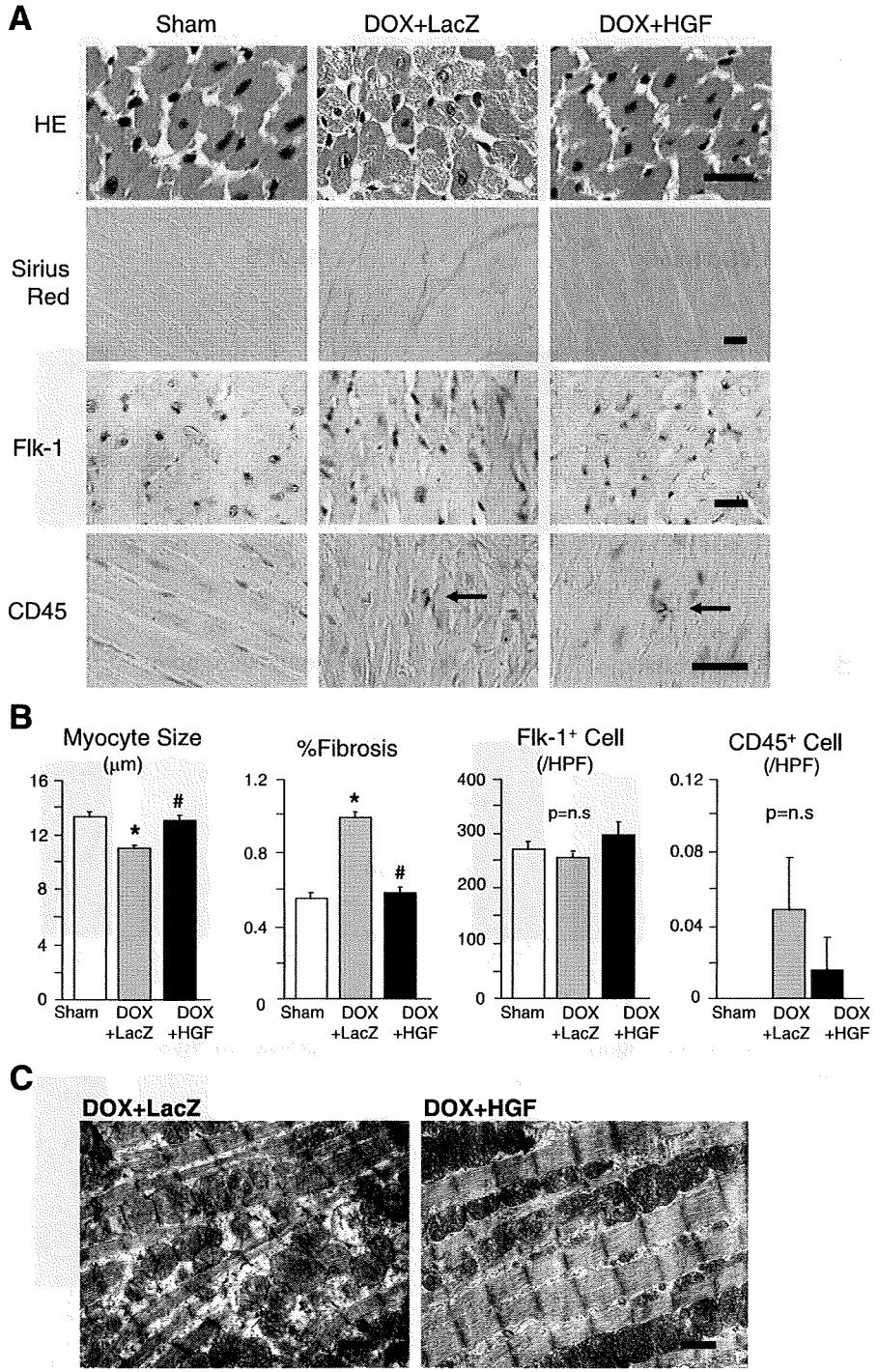

Fig. 3. Effects of hHGF gene delivery on cardiac histology in mice 4 wk after Dox administration (*protocol 1*). *A*: photomicrographs of histological [hematoxylin-eosin (HE) and Sirius red stained] and immunohistochemical (Flk-1 and CD45) preparations of heart specimens from the indicated groups. Arrows point to immunopositive cells; scale bars, 20 μm. *B*: morphometric data. $^*P < 0.05$ vs. sham group; $\#P < 0.05$ vs. Dox + LacZ group. *C*: electron photomicrographs of doxorubicin-induced cardiomyopathy treated with LacZ or hHGF gene (bar, 1 μm).

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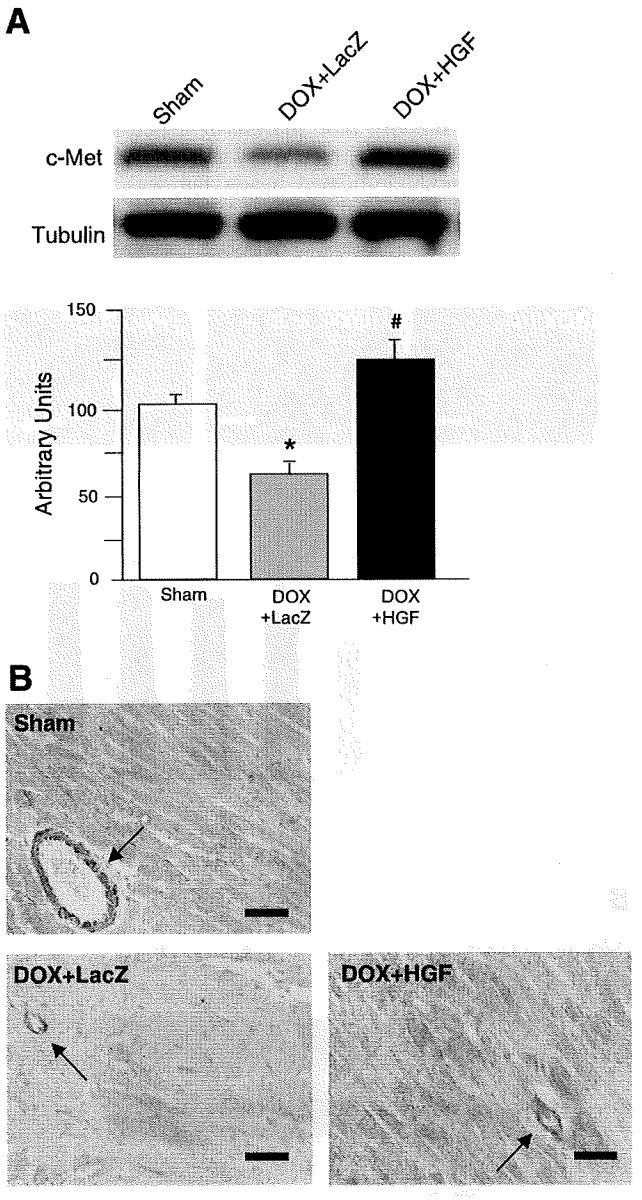

Fig. 4. c-Met expression in the heart. Western blot (*A*) and immunohistochemical (*B*) analyses. *$P < 0.05$ vs. sham group; #$P < 0.05$ vs. Dox + LacZ group. Arrows indicate intramyocardial vessels. Bars, 20 μm.

Statistical analysis. Values are shown as means ± SE. Survival was assessed by constructing Kaplan-Meier curves, which were analyzed using the log-rank Cox-Mantel method. The significance of differences between groups was evaluated using one-way ANOVA with a post hoc Newman-Keuls multiple comparisons test. Values of $P < 0.05$ were considered significant.

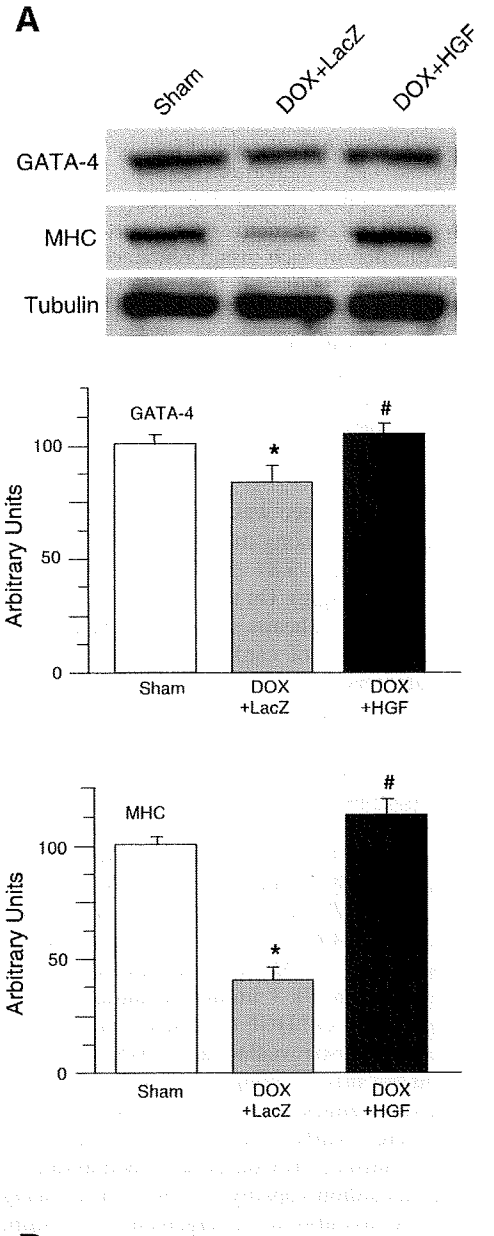

Fig. 5. Western blot analysis of sarcomere-related (*A*) and fibrosis-related (*B*) proteins. *A*: myocardial expression of GATA-4 and myosin heavy chain (MHC). *B*: myocardial expression of transforming growth factor-β1 (TGF-β1) and cyclooxygenase-2 (COX-2). Graphs are not shown for TGF-β1 and COX-2 since there were no significant differences among the groups. *$P <$ 0.05 vs. sham group; #$P < 0.05$ vs. DOX + LacZ group.

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RESULTS

hHGF in plasma and tissues. In the hindlimb receiving the adenoviral vector (Ad.CAG-HGF), hHGF levels peaked at 4.35 ± 0.03 ng/mg 3 days after injection; no hHGF was detected in the hindlimbs of LacZ-treated mice (Fig. 1*A*). Plasma hHGF also peaked 3 days after injection of Ad.CAG-HGF (3.25 ± 0.85 ng/ml), and significant levels were sustained for an additional 9 days thereafter (Fig. 1*B*). Myocardial hHGF levels showed a similar pattern (Fig. 1*C*).

Effects of hHGF gene delivery on cardiac function and pathology. All mice in each group remained alive 4 wk after doxorubicin administration. Echocardiography and cardiac catheterization showed that, compared with the saline-treated controls, mice receiving doxorubicin had significant deterioration of left ventricular (LV) functionality characterized by an enlargement of the LV cavity and decreased LV fractional shortening and ±dP/d*t* (Fig. 2). The delivery of the hHGF gene significantly attenuated the doxorubicin-induced impairment of cardiac function.

No significant difference was observed in the heart weight-to-body weight ratios among the groups (saline, 3.78 ± 0.01; doxorubicin with LacZ, 3.87 ± 0.01; and doxorubicin with hHGF, 3.71 ± 0.01 mg/g). On the other hand, an examination of transverse sections of hearts stained with hematoxylin-eosin revealed that the sizes of cardiomyocytes (expressed as the transverse diameters) from the group receiving doxorubicin plus LacZ were significantly smaller than those in the saline group (11.5 ± 0.22 vs. 13.8 ± 0.37 μm, $P < 0.05$) and that hHGF delivery exerted a significant protective effect against such doxorubicin-induced cardiomyocyte atrophy (transverse diameter, 13.4 ± 0.18 μm) (Fig. 3). Similarly, when we assessed myocardial fibrosis using Sirius red-stained sections, we found significantly greater fibrosis in the group receiving doxorubicin plus LacZ than in groups receiving saline (0.99 ± 0.05% vs. 0.55 ± 0.04%, $P < 0.05$) or doxorubicin plus hHGF (0.58 ± 0.04%) (Fig. 3). Myocardial capillary density, which we assessed based on Flk-1 immunostaining, was unaffected by either doxorubicin or hHGF treatment (Fig. 3). Immunohistochemical analysis also revealed that CD45-positive leukocyte infiltration did not differ among the groups (Fig. 3).

Degenerative changes within cardiomyocytes caused by doxorubicin were clearly evident under an electron microscope, which confirmed previously described findings in doxorubicin-induced cardiomyopathy (16, 30). These changes were characterized by myofibrillar derangement and disruption and by increases in the volume of subcellular organelles such as mitochondria (Fig. 3). These degenerative changes were significantly mitigated by hHGF gene transfer. No apoptotic cells were ever detected by electron microscopic observation of cardiac tissue from any of the groups.

TUNEL-positive cardiomyocytes were detected, though very rarely, and the incidence was not affected by either doxorubicin administration or hHGF gene transfer (saline, 0.04 ± 0.03%; doxorubicin plus LacZ, 0.06 ± 0.03%; and doxorubicin plus hHGF, 0.05 ± 0.04%). Consistent with that finding, the active (cleaved) form of caspase-3 was not detectable in hearts from any of the groups by Western blot analysis (data not shown). The absence of apoptotic cells in the present model confirms earlier studies (16, 17). In addition, proliferating cardiomyocytes, as indicated by the presence of Ki-67, were never detected (data not shown).

Expression of c-Met/HGF receptor. The HGF receptor has been identified as c-Met, the product of the c-Met proto-oncogene (5, 6). Western blot analysis revealed that the expression of the c-Met/HGF receptor was significantly downregulated in doxorubicin-treated hearts but was greatly enhanced by hHGF gene transfer (Fig. 4*A*). Consistent with this

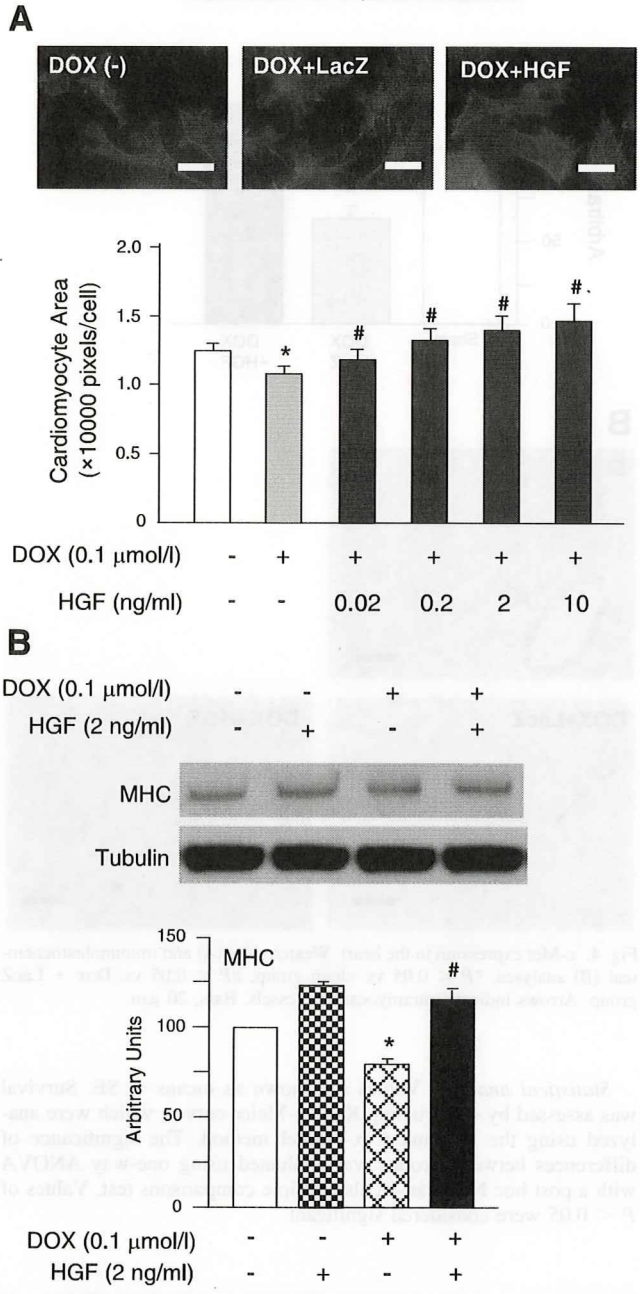

Fig. 6. In vitro experiments. *A*: confocal micrographs and a graph showing the atrophic degeneration of cardiomyocytes exposed to Dox and its prevention by recombinant hHGF. Bars, 10 μm. *$P < 0.05$ vs. sham group; #$P < 0.05$ vs. Dox + LacZ group. *B*: Western blot analysis of the effect of hHGF on the Dox-mediated reduction of MHC in cultured cardiomyocytes. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. group treated with Dox alone.

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finding, immunohistochemical analysis showed c-Met to be expressed on cardiomyocytes and to be more strongly expressed in hHGF-treated hearts (Fig. 4*B*).

Expression of GATA-4 and MHC. GATA-4 is a key transcriptional factor-regulating expression of sarcomeric proteins in the heart (22, 23). Myocardial levels of GATA-4 were significantly reduced by doxorubicin, confirming earlier reports (4). This reduction was significantly reversed by hHGF gene transfer (Fig. 5*A*). Likewise, the level of MHC was significantly reduced by doxorubicin, and this inhibitory effect was also significantly reversed by hHGF gene therapy (Fig. 5*A*).

Expression of TGF-β1 and cyclooxygenase-2. Doxorubicin had no significant effect on the expression of TGF-β1 or cyclooxygenase-2 in hearts 4 wk after administration, and neither was affected by hHGF gene transfer (Fig. 5*B*, data not shown).

In vitro effect of hHGF on cardiomyocytes. Doxorubicin exerted a significant atrophic/degenerative effect on cultured neonatal mouse cardiomyocytes, but this effect was largely reversed by an application of recombinant hHGF (Fig. 6*A*). hHGF affected the cardiomyocytes in a dose-dependent manner. Western blot analysis revealed that doxorubicin significantly reduced expression of MHC in cultured cardiomyocytes, but the expression was restored by the addition of hHGF to the cultures (Fig. 6*B*).

ERK activity. ERK/MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt are known to be components of major signaling pathways downstream of c-Met/HGF receptor (9, 24). Neither doxorubicin-induced cardiomyopathy nor the effects of hHGF gene transfer was found to be related to the activation (phosphorylation) of Akt in the heart 4 wk after doxorubicin treatment (Fig. 7*A*). In contrast, ERK phosphorylation, and thus its activation, was markedly diminished by doxorubicin,

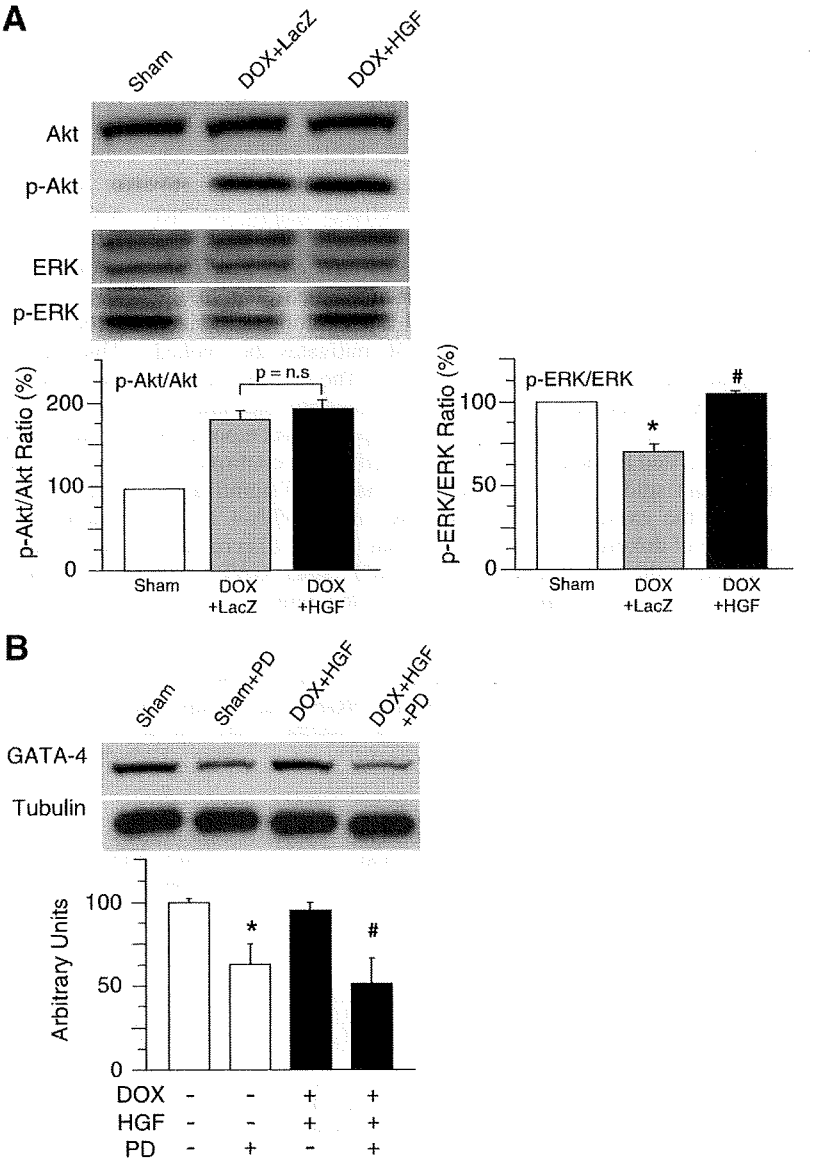

Fig. 7. *A*: Western blot analysis of the effects of hHGF gene transfer on myocardial expression of phosphorylated (p)-Akt and p-ERK. Activity of Akt and ERK is expressed, respectively, as the p-Akt-to-total Akt and p-ERK-to-total ERK ratio. *$P < 0.05$ vs. sham group; #$P < 0.05$ vs. the Dox + LacZ group. *B*: effect of the p42/p44 MAPK inhibitor PD-98059 (PD) on Dox-induced cardiomyopathy (*protocol 2*). Western blot and densitometric analyses of the effect of PD on myocardial expression of GATA-4. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. Dox + HGF group.

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and that effect was significantly attenuated by hHGF treatment (Fig. 7*A*).

To further examine the role played by ERK activation in mediating the cardioprotective effects of hHGF, we next tested the effect of inhibiting ERK activation using the MEK1-p42/p44 MAPK-specific inhibitor PD-98059 (*protocol 2*). When administered to mice along the hHGF gene, PD-98059 suppressed the hHGF-mediated reversal of doxorubicin's inhibition of GATA-4 expression (Fig. 7*B*). Moreover, PD-98059 significantly suppressed the HGF-mediated improvement in cardiac function and histology, i.e., the increase in cardiomyocyte size and the reduction in myocardial fibrosis (Table 1). This suggests that the ERK pathway is critically involved in the protective effect exerted by hHGF against doxorubicin-induced cardiomyopathy.

DISCUSSION

The present study provides clear evidence of the beneficial effects of HGF gene delivery on the cardiac dysfunction associated with doxorubicin-induced cardiomyopathy, a nonischemic cardiomyopathy. The principal pathological findings were that HGF prevented doxorubicin-induced atrophic degeneration of cardiomyocytes and myocardial fibrosis. The mechanism of action of HGF in this model differs from that seen in cases of myocardial infarction, where HGF reportedly enhances the survival of ischemic cardiomyocytes (27, 36). Notably, HGF exerted its therapeutic effects despite the fact that the cardiomyopathy was well established.

Mechanisms underlying the cardioprotective effects of HGF. Our findings suggest that several factors contribute to the cardioprotective effects of HGF against doxorubicin-induced cardiomyopathy. The first is that HGF mitigates the evoked atrophic degeneration of cardiomyocytes. The sarcomeric protein MHC is important for the structural integrity and function of cardiomyocytes, and its myocardial expression is reportedly downregulated by doxorubicin (11), an effect we confirmed in the present study. Our new finding is that HGF significantly restored the expression of both MHC and GATA-4 in the presence of doxorubicin. We suggest that GATA-4 is crucially involved in the antiatrophic effect of HGF, since GATA-4 is known to be a key regulator of heart development, to regulate myocardial expression of MHC and troponin I (22, 23) and to be depleted in doxorubicin-induced cardiotoxicity (4). Our results not only confirm those earlier findings but also demonstrate that HGF restores GATA-4 expression, even in the presence of doxorubicin.

c-Met/HGF receptor signaling is known to activate ERK/MAPK and PI3K/Akt signaling pathways (9, 24), both of which are implicated in myocardial hypertrophy (2, 5). Our findings suggest that altered signaling via ERK, but not Akt, is involved in doxorubicin-induced cardiomyopathy, which is consistent with a recent study showing that ERK activation is significantly diminished during the chronic stage of doxorubicin-induced cardiomyopathy (3 wk after doxorubicin administration) (20). Given that another study, in which isolated rat heart was subjected to excessive LV wall stress (induced by balloon inflation), showed MAPK (p38 and ERKs) to be involved in the activation of GATA-4 binding to DNA (35), we suggest that HGF exerts its cardioprotective effects by restoring activity in ERK/MAPK signaling pathway.

The HGF gene therapy significantly restored the doxorubicin-induced decrease in c-Met/HGF receptor expression in the heart, which is compatible with previous findings (18, 27): the increase in c-Met may be related to the autoinduction of gene expression triggered by HGF (27). However, immunohistochemistry showed cytoplasmic staining although c-Met is a membrane protein. One possible explanation for this discrepancy is the thickness of the sections (4 μm) relative to myocyte size (12 μm). A second possible explanation is the diffusion of diaminobenzidine products during the staining procedure. It is also possible that cytoplasmic staining is not an artifact but rather represents an abnormal distribution of excessive protein. Thus further studies are desirable in the future on the subcellular localization of c-Met in cardiomyocytes at the electron microscopic level.

Recent findings suggest that apoptosis among cardiomyocytes is a leading cause of cardiac dysfunction in doxorubicin-induced cardiomyopathy (13, 36). This hypothesis remains controversial, however, because the cardiomyocytes in question do not show the typical apoptotic morphology (16, 17, 30, 38). Seeking evidence of doxorubicin-induced apoptosis/cell death, we previously conducted a series of TUNEL assays,

Table 1. *Effects of inhibiting ERK activity with PD-98059 on LV function and histology 4 wk after administering saline or doxorubicin followed by LacZ or human HGF gene therapy: protocol 2*

	Sham (*protocol 1*)	Sham + PD-98059	Dox + HGF (*protocol 1*)	Dox + HGF + PD-98059
n	11	7	9	7
Function				
LVDd, mm	3.78±0.12	3.79±0.03	3.77±0.10	3.93±0.09
LVFS, %	29.2±1.61	30.7±0.42	25.2±1.08	20.9±0.96*
+dP/d*t*, mmHg/s	7,708±845	6,596±1,075	8,27±936	5,012±607*
−dP/d*t*, mmHg/s	−6,568±364	−6,355±976	−8,524±718	−5,434±779*
LVSP, mmHg	80.5±2.21	73.5±5.40	93.9±4.36	70.4±5.85*
Heart rate, beats/min	512±37	523±60	520±36	492±24
Histology				
Myocyte size, μm	13.8±0.37	13.5±0.20	13.4±0.18	12.4±0.32*
Fibrosis, %	0.55±0.04	0.48±0.04	0.58±0.04	0.69±0.01*
Flk-1$^+$ vessels/HPF	279±37.9	272±23.2	306±60.4	272±31.8
CD45$^+$ cells/HPF	0±0	0±0	0.02±0.04	0±0

Values are means ± SE; *n*, number of animals/group. Dox, doxorubicin; HGF, hepatocyte growth factor; LVDd, left ventricular (LV) end-diastolic diameter; LVFS, LV fractional shortening; ±dP/d*t*, maximum and minimum first derivative of pressure; LVSP, LV peak systolic pressure; HPF, high-power field. *$P < 0.05$ vs. corresponding group without PD-98059 treatment.

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electron microscopic examinations, and analyses of myocardial caspase-3 activation in the same animal model, but we detected no effect of doxorubicin on the incidence of apoptosis/cell death (17). We have now confirmed those findings. In the present study, mice received a single dose of doxorubicin, and the survival rate was 100% in all groups. This suggests the doxorubicin insult may have been too weak to induce cardiac cell death and weaker than the insults induced in earlier models. This may also hold true for our in vitro model.

HGF has been reported to be angiogenic (18, 28, 34), but we detected no doxorubicin-induced reduction in capillary density, nor did HGF promote capillary outgrowth, indicating that angiogenesis likely plays no mechanistic role in doxorubicin-induced cardiomyopathy or the cardioprotective effects of HGF.

Limitations of the study. We observed that doxorubicin stimulates the development of myocardial fibrosis and that HGF suppresses this pathological process. Although TGF-β1 is a potent stimulator of fibrosis in the failing heart, its involvement in doxorubicin-induced cardiomyopathy was challenged in a recent report (19). Consistent with that report, we found no significant doxorubicin-induced changes in the expression of TGF-β1. Therefore, although several studies suggest the mechanism underlying the antifibrotic effect of HGF is related, at least in part, to the inhibition of TGF-β1 secretion (28, 34), in the case of doxorubicin-induced cardiomyopathy, HGF appears to diminish fibrosis via a different mechanism. It is also known that doxorubicin induces cardiac expression of cyclooxygenase-2 (1), which occupies a central position in the biosynthesis of proinflammatory prostaglandin E_2, prostacyclin and thromboxane A_2, and that inhibition of cyclooxygenase-2 improves cardiac function in a model of doxorubicin-induced cardiomyopathy (10). Actually, we previously observed expression of cyclooxygenase-2 to be upregulated 2 wk after doxorubicin injection, but that is a more acute stage than the one studied here (16, 17). We did not see greater expression of cyclooxygenase-2 in the present 4-wk model, where significant infiltration of inflammatory cells also was not seen. Still, we cannot exclude the possibility that cyclooxygenase-2 contributes to the etiology of myocardial fibrosis in doxorubicin-induced cardiomyopathy. Our results also indicate that ERK inhibition blocks the antifibrotic effect of HGF in the present model; thus, further investigation will be needed to precisely define the mechanisms operating.

HGF reportedly exerts myocardial regeneration by mobilizing bone marrow-derived cells to the myocardium (15), and cardiac stem cells reportedly express c-Met/HGF receptors (12, 37). Although we did not directly evaluate the contribution made by cardiomyocyte regeneration (either from bone marrow cells or cardiac stem cells) to the beneficial effects of HGF, our immunohistochemical analysis of Ki-67, which showed an absence of cardiomyocyte proliferation, suggests that it is unlikely that cardiomyocyte regeneration plays a role in the present model. This result of ours seems to be in contrast with the previous study by Iwasaki et al. (12), which reported enhanced cardiomyocyte proliferation and increased Sca1-positive cardiac progenitor cells in doxorubicin-induced cardiomyopathy by a specific delivering method of HGF (intravenous injection of HGF delivered by ultrasound-mediated destruction of microbubbles). In addition, the peak plasma HGF concentration should have been widely different between the studies. Iwasaki et al. (12) intravenously gave 10 μg of HGF per animal (~20 g body wt), whereas in our study the plasma HGF concentration attained 3 days after gene delivery was 3.25 ± 0.85 ng/ml. These methodological differences might have a strong bearing on the different observations between the studies. Further studies are needed to focus specifically on the biological effect of HGF on stem cells.

Conclusion. The present study provides the first evidence of the beneficial effects of HGF gene transfer in doxorubicin-induced cardiomyopathy. These effects include the attenuation of atrophic degeneration of cardiomyocytes and the reduction of myocardial fibrosis, accompanied by the restoration of myocardial expression of GATA-4 and sarcomeric proteins. Our findings also suggest that HGF-mediated ERK activation is associated with these beneficial effects and may thus underlie the cardioprotection provided by HGF gene transfer.

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