研究成果の刊行に関する一覧表レイアウト（参考）

書籍

	著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
①	高橋知之、小戝健一郎	細胞増殖因子と再生医療（HGF　急性／劇症肝炎）	高木彰史	細胞増殖因子と再生医療	メディカルレビュー社	大阪	2006	*In press*

雑誌

	発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
②	Khai NC, Takahashi T, Ushikoshi H, Nagano S, Yuge K, Esaki M, Kawai T, Goto K, Murofushi Y, Fujiwara T, Fujiwara H, Kosai K.	In vivo hepatic HB-EGF gene transduction inhibits Fas-induced liver injury and induces liver regeneration in mice: A comparative study to HGF.	*J Hepatol*		*in press*	2006
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Ⅳ. 研究成果の刊行物・別刷

（収載論文 15 冊）

7. HGF

急性／劇症肝炎

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I 急性・劇症肝炎とHGF

肝炎ウイルスの感染で生じる急性肝炎は，わが国だけでも毎年約20万人の発症がみられる。急性肝炎の多くは予後良好であるが，安静，栄養補給で自然治癒を待つ以外に，病気の本体を根本的に治療（つまり病気の進展を止め，治癒再生を促進）する医薬はいまだなく，1～2％の患者は劇症肝炎へと進行してしまう。劇症肝炎は，急激な広汎性肝細胞死（肝炎ウイルスによる急性肝炎が最多の成因だが，薬剤性肝炎，自己免疫性肝炎などの急性肝障害もある）によって，肝性脳症をはじめとする肝不全症状が短期間（8週間以内）に生じる予後不良の難治性疾患であり，わが国での年間の発症数は約1,000人と推定されている。劇症肝炎の根幹の病態は急激に生じる（亜）広汎性肝細胞死であるが，現在の内科的治療法は，全身管理，合併症対策，人工肝補助という対症療法にすぎないため，その救命率も依然30～40％（亜急性型では10～20％）と予後不良であり，諸外国では肝移植が唯一の効果的治療法とされている。つまり，劇症肝炎，またその前段階の急性肝炎やその他の急性肝障害に対して，これらの病気の本態である肝細胞死の進展を阻止する根治医薬の開発は，先端医学の最重要課題の一つである。

さてHGF（hepatoctyte growth factor）は，肝再生の本体の因子として1984年に単離され，1989年にクローニングされた。血清HGF値は，健常者に比べ，急性肝炎や慢性肝炎患者では約2倍，劇症肝炎患者では約60倍の上昇がみられ，血清HGF値の上昇は肝性脳症の発症や死亡率の増加と正の相関がみられることから，予後判定の有用なマーカーとして臨床で利用されている。HGFが血清中で上昇している肝炎ほど予後が悪いという現象は一見矛盾しているかのようにみえ，また特に血清HGF値が上昇している劇症肝炎患者に外因性のリコンビナントHGFを投与し

ても何ら効果がないのではないか，という疑問も生じるかもしれない。しかし，この現象は実は，肝細胞消失に対する生体適応反応としてのHGFの産生増加と，肝細胞の障害，細胞死によるHGFクリアランスの減少を反映しているものである。さらに，劇症肝炎患者の血清中で上昇しているHGFは，必ずしも活性をもつ二本鎖HGFだけではなく，むしろ一本鎖の不活性型HGFや，生理活性はないが受容体結合能をもち活性型のHGFを阻害し得る断片化したHGFを多分に検出していることが示されている。よって，急性肝炎，急性肝障害，劇症肝炎において，活性を阻害するこのような不活性化HGFに競合し得るだけの多量の活性型HGFを人為的に補うことで，本来の生理的効果を回復するという治療法は，明確な理論的根拠をもつものである。

II 急性・劇症肝炎に対するHGFの治療効果とそのメカニズム

HGFの治療因子としての可能性は，四塩化炭素（CCl_4）やα-ナフチルイソチオシアネート（ANIT）による薬剤性肝障害の動物モデルにて，まず確認された。一方，臨床的には発症頻度も多くより重要なのは，急性肝炎，劇症肝炎に対する治療薬の開発であるが，これに関しては筆者らがその治療効果・作用と分子メカニズムを明らかにしてきた。

さて，ウイルス学的，免疫学的研究に加え，1990年代にはアポトーシスの分子機構の研究が進み，急性肝炎，劇症肝炎も，その発症，進展の分子機構がより明らかとなってきた[1) 2)]。肝炎ウイルスは肝細胞に特異的に感染し，肝細胞内で増殖するが，ウイルス蛋白自体で細胞を障害する溶解性ウイルスとは異なり，その肝細胞障害の主な機序は細胞性免疫を介した肝細胞アポトーシスの誘導である。つまり，肝炎ウイルス抗原を表出する肝細胞に，活性化された細胞障害性T細胞（キラー細胞）が結合し，キラー細胞に表出したFasリガンドが肝細胞のFasに結合することにより，肝細胞内にはアポトーシスのシグナル伝達が起こる。その後，炎症細胞が浸潤し，活性化したマクロファージから出されるTNF（tumor necrosis factor）-αなどの複合因子が肝炎をより進展させる。このように，急性肝炎というのは元来，ウイルスを駆除するための生体防御の免疫反応ではあろうが，それが病的に過剰に急激に進展すると，不可逆性の劇症肝炎に陥るものと考えられる[1) 2)]。

まず筆者らは，急性肝炎の最初に起こる病変で，劇症肝炎の進展に必須の病態でもあるFas誘導の肝細胞アポトーシスに対するHGFの治療作用を調べるため，以下のようにFas誘導の劇症肝炎に対するHGFの治療作用を検討した[1)]。Fasリガンドと同様にFas受容体に対してアゴニスティックな作用を有する抗Fas抗体（Jo-2）をマウスへ投与すると，急激な広範性肝細胞アポトーシスによる劇症肝炎が生じる。抗Fas抗体投与後，6時間には血清GPTは約7,000 IU/L，8時間後には15,000 IU/Lまで急激に上昇し，肝組織像でも6時間後に門脈域周辺に出現した肝細胞アポトーシスは8時間後には肝小葉全体に広がり，20時間以内に80%のマウスが肝不全により死亡する。これに対して，抗Fas抗体投与の，6時間前，30分前，3時間後の計3回，リコンビナントHGFを腹腔内投与したマウスでは，血清GPT上昇，肝細胞のアポトーシスがほぼ完全に抑え

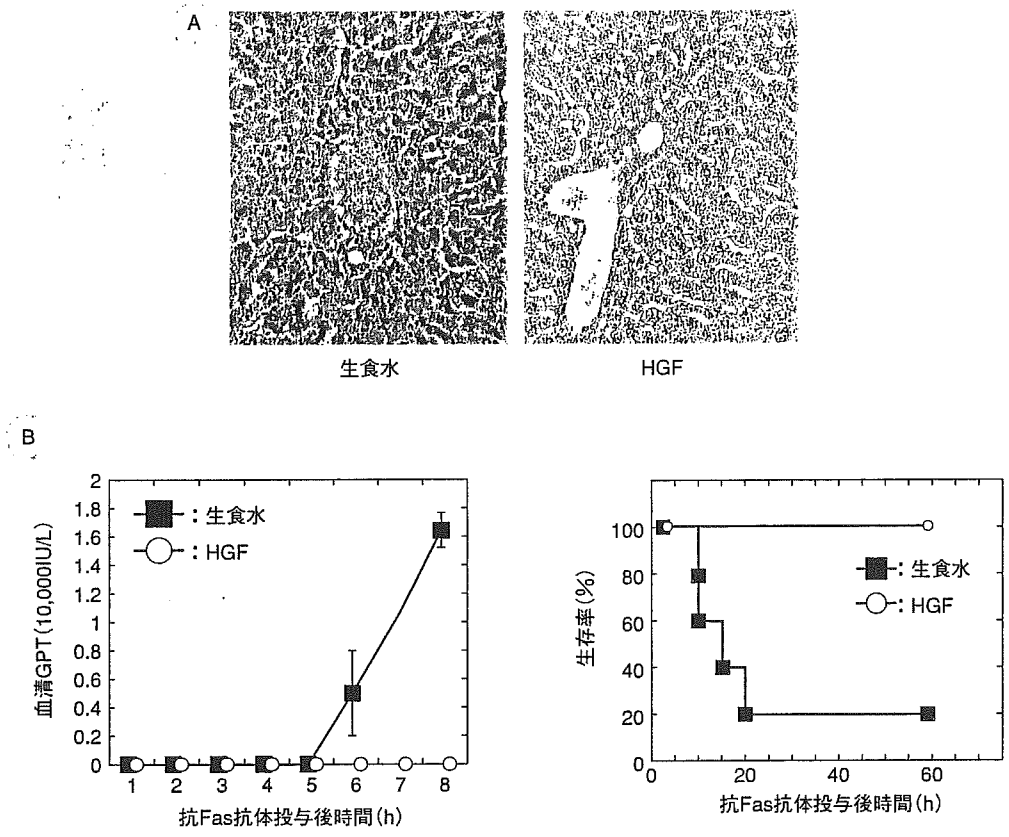

図1　HGFによる劇症肝炎の発症防止・治療効果
A：抗Fas抗体投与後24時間における肝組織像
B：抗Fas抗体投与後の血清GPT値と生存率

（文献1より引用）

られ，全マウスが生存した[1]（図1）。この結果より，HGFは，Fas誘導の肝細胞アポトーシス，劇症肝炎の発症を阻止できることが，まず明らかとなった。

さて前述のように，ヒトの劇症肝炎の原因は，肝炎ウイルス以外にも薬剤や原因不明のものもあり，またその劇症肝炎への進展の病態も，Fas誘導の劇症肝炎マウスモデルよりさらに複雑になると思われる。そこで筆者らは，TNF-αやその他の複合因子が関与し，ヒトの病態を反映するモデルとして広く用いられてきたエンドトキシン誘導劇症肝炎モデルマウスで，さらにHGFの治療作用を検討した[2]。このモデルは，細菌細胞壁成分のリポポリサッカライド（LPS）が肝マクロファージのクッパー細胞を活性化し，種々のサイトカイン放出を誘導することによって，肝細胞死を引き起こすものである。さらに，LPSと併せてその感受性を高めるD-ガラクトサミン（GalN）をマウスの腹腔内に投与すると，その5時間後より血清GPT値の急激な上昇が認められ，肝組織でも6時間後には70%以上の肝細胞が細胞死に陥り，8時間以内に全マウスが肝不全によって死亡する。一方，LPS＋GalN投与前後に計3回，HGFを腹腔内投与したマウスで

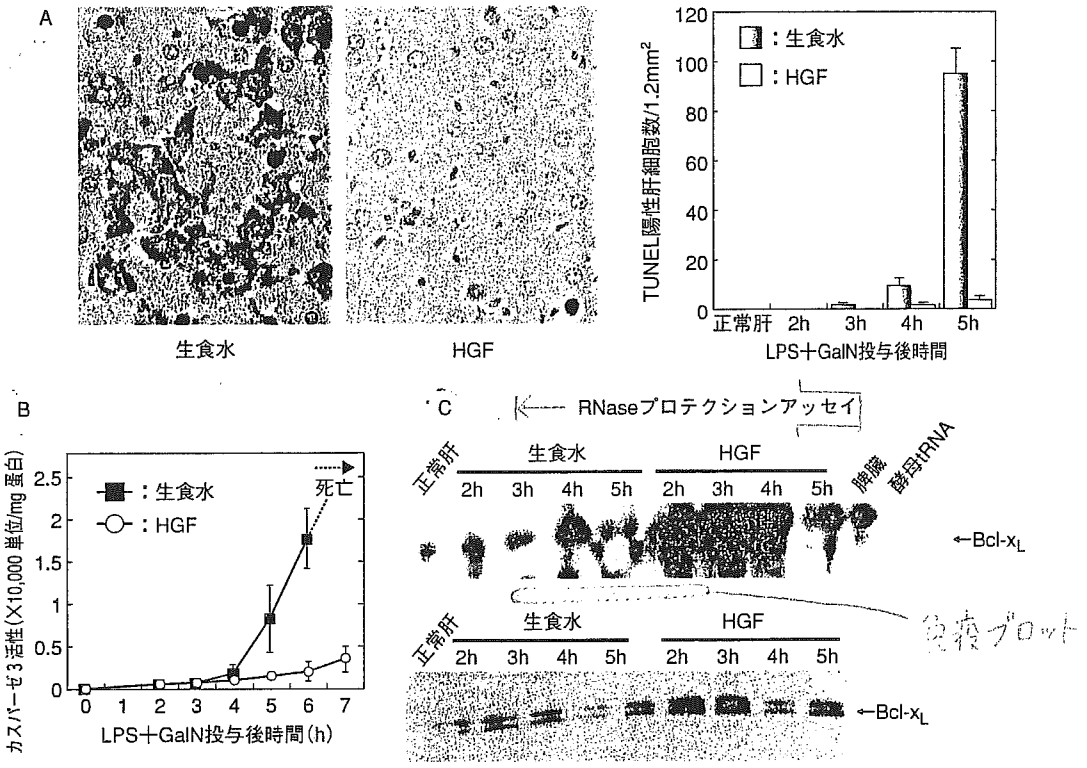

図2　HGFによる抗アポトーシス作用

A：LPS+GalN投与後5時間における肝TUNEL組織像とTUNEL陽性肝細胞数

B：LPS+GalN劇症肝炎モデルマウスに対するHGF投与による肝カスパーゼ3の活性変動

C：LPS+GalN劇症肝炎モデルマウスに対するHGF投与によるBcl-xL発現誘導

（文献2より引用）

は，血清GPT値の上昇，肝細胞アポトーシス，広範囲の肝細胞が強力に抑制され，75%ものマウスが生存した[2]。このようにHGFの治療作用は，ヒト病態を反映した複合因子による劇症肝炎モデルでも，しかも肝障害発症から3～4時間以内に肝不全によって100%死亡するというヒトの劇症肝炎とは比べ物にならないほど急激で重篤な劇症肝炎モデルでも，明確に証明された。

それではこの治療作用，分子メカニズムはいかなるものであろうか。筆者らは以下の事実から，HGFは肝細胞への強力な抗アポトーシス活性を賦与することにより，急性・劇症肝炎の病態の主因である肝細胞アポトーシスを阻止して，治療作用を生じているということを明らかにしている。まず，HGFは初代培養肝細胞に直接作用し，抗Fas抗体とアクチノマイシンDによって誘導される肝細胞アポトーシスを阻害した[1]。また，HGFは抗Fas抗体投与による純粋な*in vivo*アポトーシス誘導系のモデルと同様に，エンドトキシンモデルにおいても，TUNEL陽性肝細胞の出現，DNA断片化を強力に抑制していた[2]（図2）。さらに，Fas誘導，エンドトキシン誘導の両*in vivo*モデルの肝臓において，アポトーシスシグナルの下流に位置するカスパーゼ3の活性の急激な上昇が，HGF投与のマウス肝臓では強力に抑制されていた[1][2]。さらにHGFは同モデルの

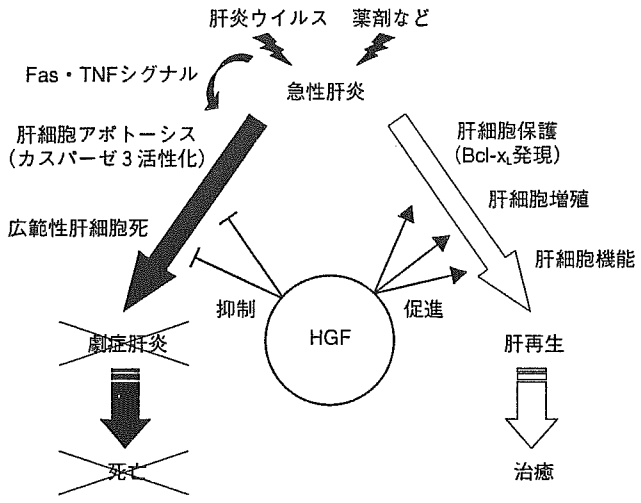

図3　HGFによる肝炎治療メカニズムの模式図

肝臓に，抗アポトーシス分子のBcl-x_L蛋白の発現を著明に誘導していた[1) 2)]。よってHGFは，肝細胞に直接作用し，Bcl-x_Lの発現誘導を介してカスパーゼ3の上流でアポトーシスのシグナル伝達を阻止し，抗アポトーシス活性を賦与しているものと考えられる。

Ⅲ　HGFを治療因子として用いる利点や今後の展望

上記の劇症肝炎マウスモデルでは，肝障害の発症から肝不全死までの全経過が2～3時間とあまりにも急激であるという実験の制約上，HGFの前投与も行った。しかし，実際のヒトの劇症肝炎患者では，急性肝炎や急性肝障害の発症から，不可逆的な肝不全に進行，死亡するまでに数日～数週間はかかるものである。よって，急性肝炎の発症後の初期にHGFを投与することは十分可能で，その段階で残存肝細胞は保護され，それ以上の肝障害の進行，劇症肝炎の発症が阻止されることが十分期待される。また，HGFは元来，肝再生の本体の因子としてクローニングされたように，同時に障害後の残存肝細胞に働き，肝再生を強力に促進する[3)]。また，HGFはアルブミンや血液凝固・線溶系関連蛋白質の合成促進作用ももち，これもさらなる治療効果として期待される。このように，HGFは急性劇症肝炎の病気の本体である肝細胞死を強力に阻止（病気の進展を阻止）し，また同時に肝障害後の肝再生を強力に誘導（病気の治癒を促進）するという，まさに急性劇症肝炎に対する理想的な根治医薬になるものと考えられる（図3）。よって，急性肝炎，薬剤性肝障害，劇症肝炎にしろ，肝障害を見つけた段階で，ともかくHGFを投与することで，これらの急性の肝疾患は治療できる（病気を止めて治癒する）ものと考えられる。

さて，その投与法であるが，生体内に静脈経由で全身投与されたHGFは，主に肝臓で補足さ

れ，血中からは速やかに（数分で）消失してしまう。よって，このような急性劇症肝炎の患者には，速攻性のあるリコンビナントHGFを，静脈経由（全身投与）で反復投与や持続点滴することが有用であると思われる。しかも，HGFは動物に有効量HGFを長期間投与しても副作用がみられないだけでなく，むしろ腎臓，肺，中枢神経，心臓に対しても組織保護・治癒作用をもつことが示されている。さらに筆者らは，安全性の面で潜在的に心配される問題で，またトランスジェニックマウスの検討では相反する結果が出ていた，肝癌に対するHGFの効果について検証し，HGF医薬は肝癌を抑制（増殖抑制とアポトーシス誘導）するという治療作用を示している[4]。この点から，予後が悪い亜急性肝炎への応用や，劇症肝炎の合併症である多臓器障害の抑制などの目的には，より長期の安定した治療濃度が得られる遺伝子治療がより有用な治療手技となる可能性もあり，筆者らもその研究を進めている[3][4]。

近年，さまざまな臓器，疾患に対し，再生医学の研究が盛んである。生後は自律再生能を消失してしまう多くの臓器の疾患の治療においては，増殖因子療法はある程度の治療効果がみられても，すでに障害され失われた組織は再生できない以上，なかなか根治にまでは至らない場合も多い。このため筆者らも，そのような臓器の代表である中枢神経や心臓に対しては，増殖因子療法に併せて，ヒトES（胚性幹）細胞などによる再建医学（細胞移植療法）の開発も進めている[5]。しかし，究極の再生療法というのは，本来生体に備わっている自然の再生能力を賦活化することで病的状態を正常化することであり，その点で生後も再生能を保持する肝臓に対しては，HGFを中心とした増殖因子による「生体内再生療法」にこそ魅力を感じ，これこそが理想の再生療法と考えている。

本稿で述べたように，急性劇症肝炎に対する医薬としてのHGFの有用性は，研究レベルでは十分に実証されているように思われる。本医薬は，少なくとも急性肝炎，その他の急性肝障害の患者には応用され得るものであるため，わが国だけでも毎年数十万人の新規患者が対象になるものと推察される。「急性肝障害にはHGFという特効薬があるから怖くない」，「劇症肝炎は過去の病気（HGFがあるから急性肝炎から進展することはない）」という時代が，一刻も早く来る日を筆者らも強く願っている。

本稿で紹介したFas誘導劇症肝炎[1]，エンドトキシン誘導劇症肝炎[2]の研究は，大阪大学大学院医学系研究科・中村敏一教授，松本邦夫先生との共同研究として行ったものです。

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In vivo hepatic HB-EGF gene transduction inhibits Fas-induced liver injury and induces liver regeneration in mice: A comparative study to HGF

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***Background/Aims*: It is unknown whether heparin-binding EGF-like growth factor (HB-EGF) can be a therapeutic agent, although previous studies suggested that HB-EGF might be a hepatotrophic factor. This study explores the potential of hepatic HB-EGF gene therapy in comparison with HGF.**

***Methods*: Mice received an intraperitoneal injection of the agonistic anti-Fas antibody 72 h after an intravenous injection of either adenoviral vector (1×10^{11} particles) expressing human HB-EGF (Ad.HB-EGF), human HGF (Ad.HGF) or no gene (Ad.dE1.3), and were sacrificed 24 or 36 h later to assess liver injury and regeneration.**

***Results*: Exogenous HB-EGF was predominantly localized on the membrane, suggesting the initial synthesis of proHB-EGF in hepatocytes. The control Ad.dE1.3-treated mice represented remarkable increases in serum ALT and AST levels and histopathologically severe liver injuries with numerous apoptosis, but a limited number of mitogenic hepatocytes. In contrast, the liver injuries and apoptotic changes were significantly inhibited, but the mitogenic hepatocytes remarkably increased, in both the Ad.HB-EGF- and Ad.HGF-treated mice. More mitogenic hepatocytes and milder injuries were observed in the Ad.HB-EGF-treated mice.**

***Conclusions*: HB-EGF has more potent protective and mitogenic effects for hepatocytes than HGF, at least for the present conditions. In vivo hepatic HB-EGF gene transduction is therapeutic for Fas-induced liver injury.**

***Keywords*: Heparin-binding epidermal growth factor-like growth factor; HB-EGF; Hepatocyte growth factor; HGF; Growth factor; Gene therapy; Apoptosis; Fulminant hepatic failure; Fas; Liver regeneration; Adenoviral vector**

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DOCTOPIC: Liver Failure, Growth and Cancer

1. Introduction

Certain growth factors act as hepatogenic and hepatotrophic factors and play essential roles in the development and homeostasis of the liver. The hepatotrophic factors that have been well studied are hepatocyte growth factor (HGF), epidermal growth factors (EGF) and transforming growth factor-α (TGF-α); their expressions are found in the adult liver under normal physiological conditions and are drastically upregulated during liver regeneration after a partial hepatectomy or liver injury [1–5]. The knockout of any of these genes in mice led to aplasia or dysmaturation of the liver [6,7], and the overexpression of any of them in the transgenic mice accelerated the proliferation of hepatocytes after a partial hepatectomy [8]. Moreover, several animal studies have shown that HGF can be a potent therapeutic agent for liver disorders by inhibiting hepatocyte apoptosis and/or inducing liver regeneration, regardless of administration of the recombinant protein or the gene therapy strategy used [3,9–16].

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which was identified as a new member of the EGF-family of growth factors, is also expressed in the normal liver [17,18]. The biologically unique feature of this growth factor is that the membrane-anchored precursor form (proHB-EGF) is initially synthesized and subsequently cleaved at the juxtamembrane domain by a specific metalloproteinase [19–21], and the resultant soluble form (sHB-EGF) represents the potent mitogenic activity for a number of cell types [22,23]. The HB-EGF mRNA levels were rapidly increased in the nonparenchymal cells of the regenerating liver, mainly in Kupffer cells and sinusoidal endothelial cells, but not in hepatocytes, after a 70% partial hepatectomy [24] or after liver injury by hepatotoxins [25]. Interestingly, the increase in HB-EGF mRNA was more rapid than that of HGF mRNA, e.g. their maximal levels were reached at 6 and 24 h after partial hepatectomy, respectively [24], suggesting the distinct role and/or mechanism of HB-EGF in liver regeneration compared to HGF. Moreover, exogenous HB-EGF stimulated the DNA synthesis in rat hepatocytes in in vitro [25] and in vivo experiments [26], and the hepatocyte-specific overexpression of HB-EGF in the transgenic mice accelerated the in vivo proliferation of hepatocytes after a partial hepatectomy [27]. Thus, it is likely that HB-EGF may also be acting as the hepatotrophic factor, probably in the early phase of liver regeneration. However, the therapeutic potential of HB-EGF for liver disorders, e.g. the question of whether exogenous HB-EGF acts to inhibit liver injury, has not yet been studied. Other important points are that the possibility of HB-EGF gene therapy has not yet been studied for any diseases in any organs, including liver diseases, except for our recent study on heart disorders, and that the HB-EGF gene therapy trial for myocardial infarction in rabbits did not exert therapeutic effects but rather exacerbated remodeling [28]. Thus, it is biologically and clinically meaningful to investigate whether HB-EGF gene therapy can be potentially used to treat liver disorders.

In this study, the HB-EGF gene was adenovirally transduced into the mouse liver and its potential to inhibit liver injury and stimulate liver regeneration were investigated. Furthermore, these potentials were simultaneously compared to those of HGF, which is currently the best-studied hepatotrophic and therapeutic factor.

2. Materials and methods

2.1. Recombinant adenoviral vectors

Replication-defective recombinant adenoviral vectors (Ads), Ad.HB-EGF, Ad.HGF, Ad.LacZ and Ad.dE1.3, which express human HB-EGF, human HGF, LacZ and no gene under the transcriptional control of a Rous sarcoma virus long-terminal repeat, were prepared as described previously [28–31].

2.2. Animal studies

The schedule of the experiment on therapeutic potentials is shown in Fig. 2(A). Male 5 to 6 week-old C57BL/6J mice ($n=10$, each group) (Chubu Kagaku, Nagoya, Japan) were given an intravenous injection of 1×10^{11} particles of Ad via a tail vein. 72 h later, they were given an intraperitoneal injection of 4 μg of agonistic anti-mouse Fas monoclonal antibody (Jo-2, Beckton-Dickinson Biosciences, San Jose, CA) [14]. All mice were subsequently sacrificed 24 or 36 h later, and liver and blood samples were collected for examination. On the other hand, in vivo adenoviral gene transduction efficiency or the in situ detection of exogenous human HB-EGF was analyzed using the same dose (1×10^{11} particles) of either Ad.LacZ or Ad.HB-EGF, respectively, in intact mice. All animal studies were performed in accordance with the National Institute of Health guidelines as dictated by the Animal Care Facility at the Gifu University School of Medicine.

2.3. Histopathologic analysis

For histopathological observation, liver tissues were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were cut and stained with hematoxylin and eosin (H-E). For assessing the in vivo adenoviral gene transduction efficiency, *O*-nitrophenyl-β-D-galactophyranoside (x-gal) staining was done using the frozen tissue as described previously [28–31]. For detecting apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay (ApopTag kit, Intergen Co., Purchase, NY) was done in accordance with the manufacturer's protocol.

Immunohistochemistry was carried out for in situ detection of the cells expressing the HB-EGF transgene or regenerating hepatocytes. In the former, 6-μm frozen sections were fixed in 4% paraformaldehyde and stained with primary goat anti-human HB-EGF antibody (R&D Systems Inc., Minneapolis, MN), secondary donkey anti-goat IgG Alexa 568 antibody (Molecular Probes, Inc., Eugene, OR), and Hoechst 33342 (Molecular Probes, Inc.). In the latter, the formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated, and then heated in 10 mmol/L citrate buffer, pH 6.0 for 10 min for the antigen retrieval. Endogenous peroxidase activity and the non-specific binding of antibody were blocked by 0.3% H_2O_2 and normal rabbit serum. The anti-Ki67 (TEC-3, DakoCytomation, Denmark) primary antibody, biotinylated anti-rat IgG secondary antibody, avidin-peroxidase, diaminobenzidine in hydrogen peroxidase and chromogen were applied in order and were subsequently counterstained with hematoxylin.

Digital images observed with a laser-confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany) were employed for the morphometric and quantitative analyses using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA). Quantitative analyses of the replicating and apoptotic hepatocytes were done as described previously with some modifications [13,15]. Briefly, Ki-67-positive, Ki-67-negative or TUNEL-positive hepatocytes were counted in 30 fields at random under 200×

magnification (approximately 2000 cells in 3 slides per mouse), and the percentages of the replicating hepatocytes and numbers of the apoptotic hepatocytes in a field were calculated.

2.4. Biochemical Analyses

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a standard clinical automatic analyzer (Hitachi 736) (Hitachi Co. Ltd., Tokyo, Japan) at 24 or 36 h after the anti-Fas antibody injection.

2.5. Statistical Analysis

Data were represented as the means +/− standard errors. Statistical significance was determined using the Student's *t*-test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Adenoviral gene transduction efficiency and HB-EGF transgene expression

In accordance with previous findings [16,32], Ad.LacZ injection and subsequent x-gal staining revealed that an intravenous injection of 1×10^{11} particles of Ad resulted in approximately between 60 and 90% gene transduction in hepatocytes (Fig. 1(A)). Ad.HB-EGF injection and the subsequent immunohistochemistry against the human HB-EGF confirmed this finding. Moreover, the exogenous HB-EGF protein was predominantly observed on the membrane, and the cytoplasm was faintly positive for exogenous HB-EGF. These findings suggest that the initially synthesized exogenous HB-EGF was membrane-anchored proHB-EGF, and that the resultant sHB-EGF by the shedding of proHB-EGF might bind to and activate hepatocytes in the autocrine fashion, followed by the endocytosis of exogenous HB-EGF in hepatocytes.

3.2. Liver enzymes after HB-EGF or HGF gene therapy

Recent studies have suggested that the initial and essential event in acute and/or chronic hepatitis, including fulminant hepatic failure, may be the excessive activation of the Fas system [33,34] and that the administration of the agonistic anti-Fas antibody in mice leads to fulminant hepatic failure [14,35]. As we clearly elucidated the therapeutic effect of HGF in this animal model [14], this model may be suitable to use for the initial examination of the in vivo anti-apoptotic effect on hepatocytes and the

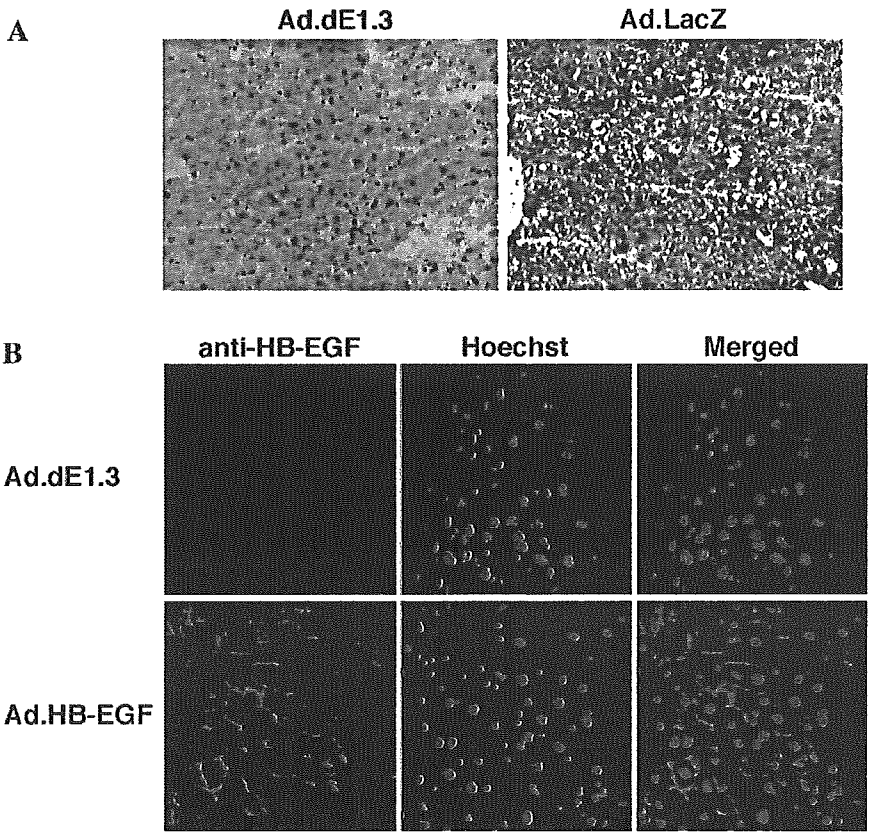

Fig. 1. Adenoviral gene transduction and expression in mice. (A) X-gal staining of the liver 48 h after an intravenous injection of either Ad.LacZ or control Ad.dE1.3 into an intact mouse. (B) Immunohistochemical staining for exogenous human HB-EGF of the liver 48 h after an intravenous injection of either Ad.HB-EGF or control Ad.dE1.3 into an intact mouse.

therapeutic potential of a certain agent for liver disorders. To perform this assessment, we initially injected Ad, and then subsequently injected the anti-Fas antibody 72 h later. We sacrificed the mice 24 or 36 h after the anti-Fas injection and examined the hepatic injuries (Fig. 2(A)).

The mice that received injections of the control Ad.dE1.3 and anti-Fas antibody represented a remarkable increase in the serum ALT and AST levels, which were up to 2240±450 and 1665±391 IU/L (62 and 21 times as high as the normal levels), respectively, 24 h after the anti-Fas injection (Fig. 2(B) and (C)). The increases in the serum ALT and AST levels were significantly attenuated by an injection of either Ad.HB-EGF or Ad.HGF before the anti-Fas injection ($P<0.01$); the serum ALT and AST levels were similar (i.e. no statistically significant differences between these two groups) and less than 230 IU/L in both groups. On the other hand, the serum ALT and AST levels 36 h after the anti-Fas injection in the Ad.HB-EGF-treated mice were remarkably lower than those in not only the control Ad.dE1.3-treated but also the Ad.HGF-treated mice. The serum ALT and AST levels in the Ad.HB-EGF-treated mice were 12 and 8.4 times (at 24 h) and 2.0 and 3.1 times (at 36 h) as low as those in the control Ad.dE1.3-treated mice, respectively. Those in the Ad.HGF-treated mice were 10 and 8.1 times (at 24 h) and 0.7 and 0.9 times (i.e. no protective phenotype) (at 36 h), as low as those in the control Ad.dE1.3-treated ones, respectively. Thus, these results indicate that HB-EGF has a more potent inhibitory effect on Fas-induced liver injuries than HGF, at least for the present condition.

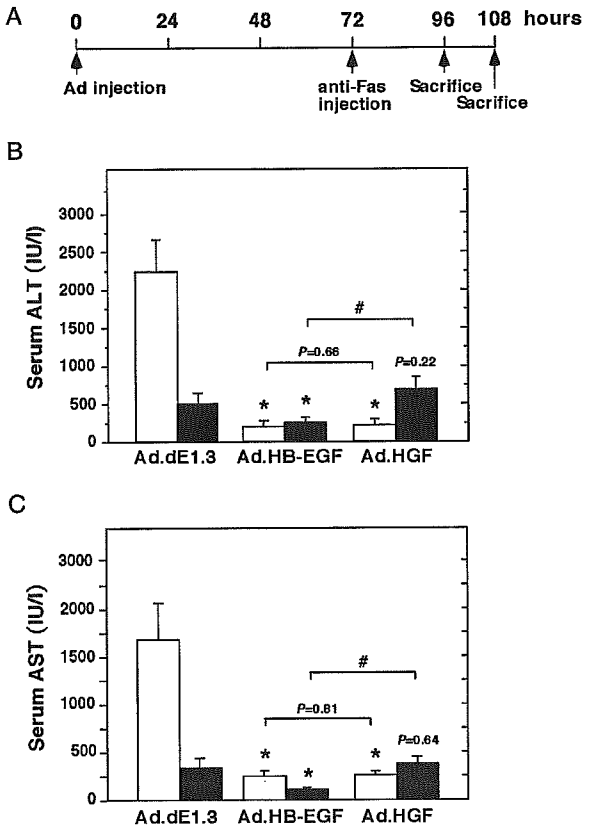

Fig. 2. Experimental schedule and liver enzymes after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) Experimental schedule of all the following therapeutic experiments. Mice ($n=10$, each group) received intraperitoneal injections of 4 μg of agonistic anti-Fas antibody 72 h after a tail vein injection of either Ad.hHB-EGF, Ad.hHGF or Ad.dE1.3 (1×10^{11} particles). The mice were then sacrificed, and liver and blood samples collected 24 or 36 h after injection of the anti-Fas antibody (i.e. 96 or 108 h after adenoviral injection). The serum ALT and AST levels at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody are shown on (B) and (C), respectively (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

3.3. Liver histopathology after HB-EGF or HGF gene therapy

The histological analysis of the livers at 24 and 36 h after the anti-Fas antibody injection in the control Ad.dE1.3-treated mice demonstrated severe liver injury with prominent apoptotic changes, as described previously [14,15] (Fig. 3). Briefly, apoptotic bodies characterized by nuclear and cell fragmentation within the shrunken and condensed cytoplasm were found, and there were a number of cells, consisting of spindle-shaped activated Kupffer cells, mononuclear cells and neutrophils, in the sinusoid. Such findings have often been observed in human patients with liver injuries as the apopto-necro-inflammatory reaction [33,36].

In contrast, there were minimal histopathological findings in the liver in the Ad.HB-EGF- or Ad.HGF-treated mice 24 and 36 h after the anti-Fas antibody injection; the inhibitory effect against liver injury as assessed by histopathologic observation on the H-E-stained slides in these mice was seemingly much more prominent than that assessed by the serum ALT and AST levels, as shown above. Apoptotic bodies were rarely seen, and the inflammatory reaction characterized by Kuppfer cell hyperplasia and accumulated inflammatory cells was not found. The difference in the histopathology of the liver between the Ad.HB-EGF- and Ad.HGF-treated mice was not clear at 24 h, but was remarkable at 36 h after the anti-Fas antibody injection. Notably, the pathological findings were still minimal in the Ad.HB-EGF-treated mice, but relatively prominent in the Ad.HGF-treated mice at 36 h after the anti-Fas antibody. These results indicated that HB-EGF has more potent cytoprotective and inhibitory effects against the Fas-induced apopto-necro-inflammatory reaction than HGF, at least for the present condition.

3.4. Potent anti-apoptotic effects of HB-EGF or HGF gene therapy

To obtain evidence that HB-EGF prevents hepatocyte apoptosis, apoptotic cells were detected by the in situ TUNEL assay (Fig. 4). In accordance with the

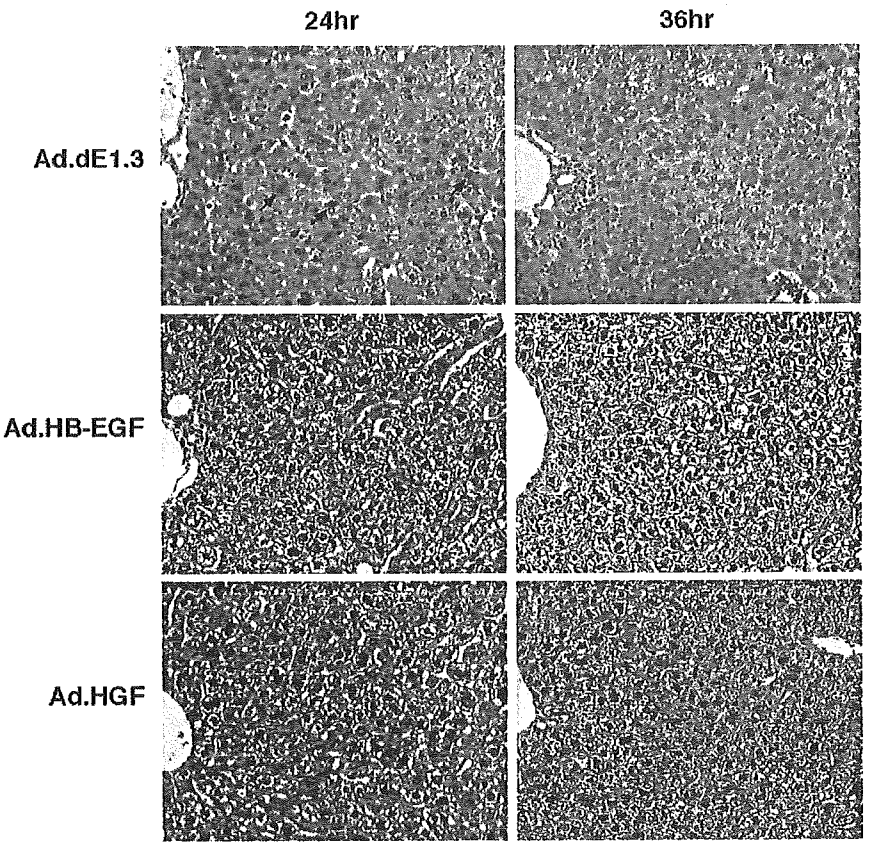

Fig. 3. Liver histopathology after HB-EGF or HGF gene therapy for Fas-induced liver injury. H-E stained liver slides of the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A) are shown. Arrows indicate typical acidophilic and apoptotic bodies. Original magnification, ×200. [This figure appears in colour on the web.]

histopathological findings on H-E slides, TUNEL-positive hepatocytes, approximately 10 or 6% of all hepatocytes, were observed throughout the liver parenchyma in the Ad.dE1.3-treated control mice 24 or 36 h after the anti-Fas antibody injection, respectively. Apoptotic bodies recognized in the disrupted hepatic cord or in the sinusoids were also TUNEL-positive. On the other hand, there were few (around 1%) TUNEL-positive hepatocytes in the liver of the mice treated with either Ad.HB-EGF or Ad.HGF at 24 and 36 h after the anti-Fas antibody injection. The morphometric and quantitative analysis of the TUNEL-positive cells revealed statistically significant differences between the control Ad.dE1.3- and either the Ad.HB-EGF- and Ad.HGF-treated groups ($P<0.01$), but no difference between the Ad.HB-EGF- and Ad.HGF-treated groups at 24 or 36 h after the anti-Fas antibody injection. These data suggest the anti-apoptotic effect of HB-EGF on hepatocytes is as potent as HGF.

3.5. HB-EGF induces liver regeneration more potently than HGF

We examined whether HB-EGF gene transduction and expression in the autocrine fashion may enhance liver regeneration after liver injury, and compared the degree of the inducible effect of HB-EGF to that of HGF by morphometric and quantitative analysis of Ki-67-positive cells (Fig. 5). In the control mice that received injections of Ad.dE1.3, Ki-67-positive replicating hepatocytes at 24 and 36 h after the anti-Fas antibody injection were 6.4 ± 0.7 and $12\pm2.2\%$, respectively, which were more than that of the intact liver without any treatment (less than 1%; data not shown). On the other hand, Ad.HGF injection significantly enhanced the hepatocyte replication at 24 and 36 h after the anti-Fas antibody up to 35.8 ± 2.8 and $28\pm3.0\%$, respectively ($P<0.01$, Ad.HGF vs. Ad.dE1.3). More promisingly, the Ad.HB-EGF injection induced the hepatocyte replication at 24 h after the anti-Fas antibody more efficiently up to $54.6\pm2.5\%$ ($P<0.01$, Ad.HB-EGF vs. Ad.HGF, and Ad.HB-EGF vs. Ad.dE1.3). The percentages of Ki-67-positive replicating hepatocytes in Ad.HB-EGF-treated mice at 36 h after the anti-Fas antibody were $29\pm2.9\%$, similar to those in the Ad.HGF-treated mice. Thus, HB-EGF gene transduction and expression in the hepatocytes induced the acute phase of liver regeneration after liver injury more potently than HGF.

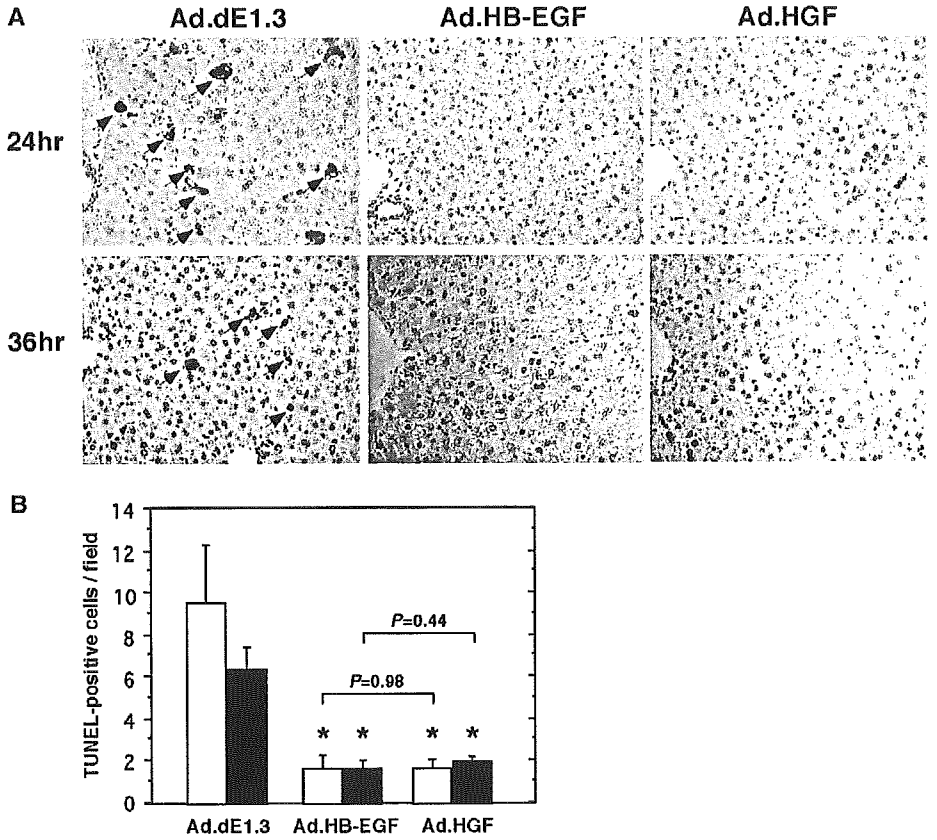

Fig. 4. TUNEL analysis of the liver after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) TUNEL staining using the liver tissue from the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A). Arrows indicate TUNEL-positive hepatocytes and apoptotic bodies. Original magnification, ×200. (B) The morphometric and quantitative analysis of TUNEL-positive cells at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody in each group (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

4. Discussion

It has been shown that exogenous HGF can be a potent therapeutic agent for liver disorders in terms of its beneficial effects of inhibiting liver injury and inducing liver regeneration, regardless of administration of the recombinant protein or the gene therapy strategy used [3,9–16]. In contrast to such extensive studies on HGF, the therapeutic potential of HB-EGF has not yet been explored. A few studies have shown the in vivo mitogenic effect of exogenous HB-EGF [26,27,37], but neither the anti-apoptotic effect on hepatocytes nor the inhibitory effect against liver injury have yet been studied. Thus, the present study revealed, for the first time, that HB-EGF might be a therapeutic agent for liver disorders in terms of both its anti-apoptotic and mitogenic effects on hepatocytes. On the other hand, we recently showed that in vivo HB-EGF gene transduction for myocardial infarction in the heart did not demonstrate therapeutic effects but instead exacerbated the remodeling, although HB-EGF is one of the essential cardiogenic and cardiotrophic factors [28]. Thus, the present results, taken together with our recent ones, are useful for understanding the physiological and pathological roles of HB-EGF. The phenotypic difference of HB-EGF may, at least in part, be due to potent and no cytoprotective effects of HB-EGF for hepatocytes and cardiomyocytes, respectively, although future extensive studies are necessary for overall elucidation.

Moreover, we obtained a further promising result in this study that the in vivo mitogenic and protective effects of HB-EGF on hepatocytes were more potent than those of HGF, at least in part and for the present condition. There are several possibilities why. The first possibility is the definitive difference between the HGF- and HB-EGF-dependent signal transduction pathways in hepatocytes related to the mitogenic phenotype; this is likely because their receptors, i.e. the c-met/HGF receptor and several EGF/erbB receptors, are different [3,20]. The second possibility is that this difference may result from the fact that HB-EGF plays more important roles in the early phase of liver regeneration than HGF. It was reported that the increase in the HB-EGF mRNA level occurred earlier than that of the HGF mRNA level during liver regeneration after partial hepatectomy or liver injury by hepatotoxins

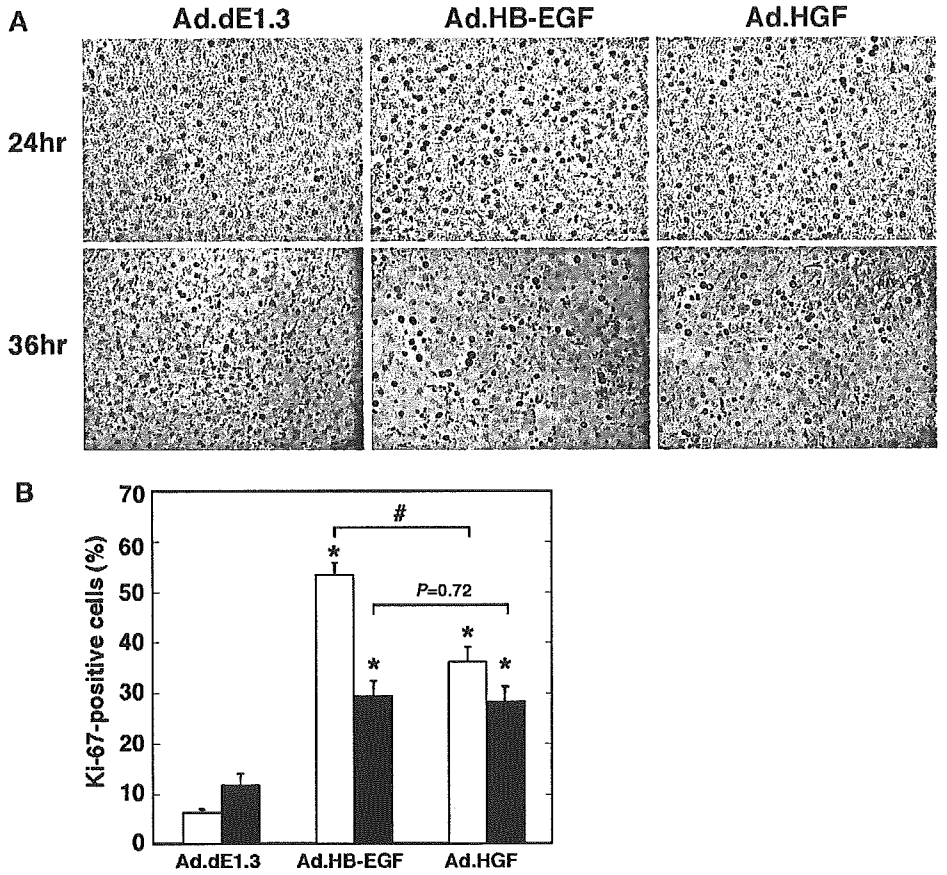

Fig. 5. Ki-67 immunohistochemistry of the liver after HB-EGF or HGF gene therapy for Fas-induced liver injury. (A) Immunohistochemistry against Ki-67 using the liver tissue from the mice that were given injections of the anti-Fas antibody and either Ad.dE1.3, Ad.HB-EGF or Ad.HGF on the protocol shown in Fig. 2(A). Original magnification, ×200. (B) The morphometric and quantitative analysis of Ki-67-positive cells at 24 (white bars) or 36 (black bars) hours after injection of the anti-Fas antibody in each group (* $P<0.01$, either Ad.HB-EGF or Ad.HGF vs. control Ad.dE1.3: # $P<0.01$, Ad.HB-EGF vs. Ad.HGF).

[24,25,38]. Another study showed that the additions of exogenous HB-EGF to nonparenchymal cells in the in vitro primary culture increased the HGF mRNA expressions in these cells, suggesting that HB-EGF may induce HGF production [24,26]. Thus, one hypothesis is that exogenous HB-EGF produced from the transduced human HB-EGF gene may sequentially activate several downstream factors, including HGF, and that these secondary effects together with the direct effect of HB-EGF may additively induce the higher mitogenic activity [39]. It would be interesting to investigate the detailed molecular mechanisms and the possibility of HB-EGF-induced liver regeneration in the case of a no priming event and the possible synergic or additive effects of HB-EGF and HGF combination gene therapy in future extensive studies.

In this study, we used an adenoviral vector and Fas-induced liver injury model solely for exploring the possibility and potential of HB-EGF gene therapy for liver diseases. A biologically important fact concerning the availability of hepatic HB-EGF gene therapy is that synthetic and proteolytic processes were reproduced even in hepatocytes, and that exogenous HB-EGF can efficiently activate hepatocytes in an artificial autocrine fashion. From the clinical viewpoint, more potent protective and mitogenic effects of HB-EGF for hepatocytes than those of HGF are apparently promising and potentially beneficial for treating liver disorders. However, it remains to be elucidated whether HB-EGF is a more clinically useful therapeutic agent for liver diseases than HGF. In actuality, some reports showed that HB-EGF was highly expressed in hepatocytes during hepatocarcinogenesis [40–42], and our recent study has shown that HB-EGF plays pathological roles in heart disorders [28]. In this regard, future studies are needed to investigate the clinical usefulness, including the possible adverse effects (e.g. fibrosis and hepatocarcinogenesis), of HB-EGF gene therapy for chronic hepatitis and liver cirrhosis, as we have carefully done in HGF gene therapy [29].

In conclusion, HB-EGF may have more potent protective and mitogenic activities for hepatocytes than HGF, at least

in part and for the present condition. In vivo HB-EGF gene transduction in the liver is therapeutic for Fas-induced liver injury.

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Bone marrow-derived myocyte-like cells and regulation of repair-related cytokines after bone marrow cell transplantation

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Abstract

Objective: Whether bone marrow cells injected following acute myocardial infarction (MI) transdifferentiate into cardiomyocytes remains controversial, and how these cells affect repair-related cytokines is not known.
Methods: Autologous bone marrow-derived mononuclear cells (BM-MNCs) labeled with DiI, 1,1′-dioctadecyl-1 to 3,3,3′,3′-tetramethylindocarbocyanine perchlorate, or saline were intravenously injected into rabbits 5 h following a 30-min ischemia and reperfusion protocol, and cardiac function and the general pathology of the infarcted heart were followed up 1 and 3 months post-MI. To search for regenerated myocardium, electron microscopy as well as confocal microscopy were performed in the infarcted myocardium 7 days post-MI. Expression levels of repair-related cytokines were evaluated by immunohistochemistry and Western blotting.
Results: Improvements in cardiac function and reductions in infarct size were observed in the BM-MNC group 1 month and 3 months post-MI. Using electron microscopy 7 days after infarction, clusters of very immature (fetal) and relatively mature cardiomyocytes undergoing differentiation were identified in the infarcted anterior LV wall in the BM-MNC group, though their numbers were small. These cells contained many small and dense DiI particles (a BM-MNC marker), indicating that cardiomyocytes had regenerated from the injected BM-MNCs. The expression of both transforming growth factor-β, which stimulates collagen synthesis and matrix metalloproteinase-1, a collagenase, were both down-regulated 7 days and 1 month post-MI in the BM-MNC group. Stromal cell-derived factor-1, which is known to recruit BM-MNCs into target tissues, was overexpressed in the infarcted areas of BM-MNC hearts 7 days post-MI.
Conclusions: Intravenous transplantation of BM-MNCs leads to the development of BM-MNC-derived myocyte-like cells and regulates the expression of repair-related cytokines that facilitate repair following myocardial infarction.

Keywords: Myocardial regeneration; Ultrastructure; Cardiac repair; Bone marrow; Cytokines

1. Introduction

Transplantation of bone marrow-derived mononuclear cells (BM-MNCs), including hematopoietic and mesenchymal stem cells, following acute myocardial infarction (MI) diminishes left ventricular (LV) remodeling, improves LV function, and reduces the size of old infarcts in post-MI hearts [1–3]. Nevertheless, the presence of BM-derived cardiomyocytes in infarcted myocardium remains controversial because although they are readily detectable in some cases [1], they are undetectable in others [4–6]. Contributing to this discrepancy may be an overestimation or underestimation of the numbers of BM-derived cardiomyo-

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