Heparin-binding epidermal growth factor-like growth factor and hepatocyte growth factor inhibit cholestatic liver injury in mice through different mechanisms

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Abstract. In contrast to hepatocyte growth factor (HGF), the therapeutic potential and pathophysiologic roles of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in liver diseases remain relatively unknown. To address the lack of effective pharmacologic treatments for cholestatic liver injuries, as well as to clarify the biologic features of these growth factors, we explored the effects of HB-EGF and HGF in mice with cholestatic liver injury induced by bile duct ligation (BDL). The mice were assessed 3, 5 and/or 14 days after BDL (acute, subacute and/or chronic phases, respectively) and intravenous injection of adenoviral vector expressing LacZ (control), HB-EGF, HGF, or HB-EGF and HGF. HB-EGF, HGF, or a combination of the growth factors exerted potent antioncotic (antinecrotic), antiapoptotic, anticholestatic, and regenerative effects on hepatocytes *in vivo*, whereas no robust antiapoptotic or regenerative effects were detected in interlobular bile ducts. Based on serum transaminase levels, the acute protective effects of HB-EGF on hepatocytes were greater than those of HGF. On the other hand, liver fibrosis and cholestasis during the chronic phase were more potently inhibited by HGF compared with HB-EGF. Compared with either growth factor alone, combining HB-EGF and HGF produced greater anticholestatic and regenerative effects during the chronic phase. Taken together, these findings suggest that HB-EGF and HGF inhibited BDL-induced cholestatic liver injury, predominantly by exerting acute cytoprotective and chronic antifibrotic effects, respectively; combining the growth factors enhanced the anticholestatic effects and liver regeneration during the chronic phase. Our results contribute to a better understanding of the pathophysiologic roles of HB-EGF and HGF, as well as to the development of novel effective therapies for cholestatic liver injuries.

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Abbreviations: HGF, hepatocyte growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Ads, adenoviral vectors; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; α-SMA, α-smooth muscle actin

Key words: adenoviral vector, cholestatic liver diseases, gene therapy, heparin-binding epidermal growth factor-like growth factor, hepatic fibrosis, hepatocyte growth factor, liver regeneration, necrosis (oncosis), apoptosis, bile duct obstruction (ligation)

Introduction

Caused by impaired bile formation or bile acid excretion from hepatocytes into the bile canaliculi, cholestasis occurs in a wide variety of human diseases, including biliary atresia, Alagille syndrome, primary biliary cirrhosis, and primary sclerosing cholangitis (1-3). The accumulation of toxic bile salt damages hepatocytes and cholangiocytes, resulting in apoptosis and oncosis (characteristic hepatocyte necrosis in response to obstructive cholestasis); together with the inflammatory reactions, the resultant cell death exacerbates liver injuries, ultimately leading to fibrosis, cirrhosis and liver failure (4-9). There is no effective pharmacologic treatment for cholestatic liver disease, and, with the exception of liver transplantation, surgical treatments are often ineffective, particularly in the advanced stages of these disorders (1,2,10). Indeed, cholestatic liver disease is best treated via liver transplantation, accounting for 24 % of all liver transplantation cases (10). Yet liver transplantation is associated with several limitations, including shortages of available donors, postoperative complications and immunologic rejection (10-12). In this regard, current efforts are focusing on innovative pharmacotherapy to attenuate cholestatic liver injuries and/or inhibit the progression of these disorders.

Certain growth factors are hepatogenic and hepatotrophic, playing essential roles in liver development and homeostasis.

One well-studied hepatotrophic factor is hepatocyte growth factor (HGF), a heterodimer composed of a 69-kDa α chain and 34-kDa β chain that was originally identified and cloned as a potent mitogen for hepatocytes (13,14). Preclinical studies in animal disease models suggest that HGF can be therapeutic for several liver disorders, including acute hepatitis, fulminant hepatic failure, chronic hepatitis, liver fibrosis and cirrhosis, and liver cancer (15-22). Moreover, three independent studies using recombinant HGF protein, naked HGF expression plasmid, or HGF-expressing adeno-associated viral vector demonstrated that HGF prevented and/or treated bile duct ligation (BDL)-induced cholestatic liver injury (23-25).

On the other hand, heparin-binding epidermal growth factor-like growth factor (HB-EGF), a new member of the epidermal growth factor (EGF) family, is expressed in normal liver tissues, and HB-EGF mRNA levels increase more rapidly than those of HGF after liver injury (26,27). The unique feature of HB-EGF is that its membrane-anchored precursor form (proHB-EGF) is initially synthesized and subsequently cleaved at the juxtamembrane domain by a specific metalloproteinase; the resultant soluble form (sHB-EGF) acts on certain cell types, including hepatocytes, to induce mitogenic and regenerative activities (28-31). In contrast to HGF, which has been extensively studied, the potential therapeutic effects of HB-EGF remain controversial. For instance, a study using HB-EGF-null mice demonstrated a suppressive effect of HB-EGF in experimental liver fibrosis (32), whereas other studies have suggested that HB-EGF induces fibrosis (33-35). This fact, together with historical lessons from the opposing results of HGF on hepatocarcinogenesis using different transgenic mouse models (36,37), suggests that a direct assessment of therapeutic potential by the administration of the HB-EGF agent is crucial in order to come to a definitive conclusion. In addition, simultaneous comparison of the therapeutic effects of the HB-EGF agent to those of the well-characterized HGF agent in the same disease model should provide a great deal of information about the differences between these growth factors with regard to their biologic roles and therapeutic effects. Our previous studies have revealed that treatment of mice with HB-EGF was effective against acute liver injury and lethal fulminant hepatic failure caused by Fas-mediated hepatocyte apoptosis (29,30); in these models, HB-EGF produced greater protective and mitogenic effects in hepatocytes than HGF (29). However, the therapeutic effects of HB-EGF in other liver disorders - particularly, its potential inhibition of hepatocyte necrosis, liver fibrosis and cholestasis - have not yet been elucidated.

In this study, the HB-EGF and/or HGF genes were adenovirally transduced into the livers of mice subjected to BDL. We assessed liver regeneration and inhibition of liver injuries, including the apoptosis and oncosis (necrosis) of hepatocytes, cholestasis and liver fibrosis. This study elucidates not only that HB-EGF is therapeutic for cholestatic liver injury but also that HB-EGF exerts potent antinecrotic (antioncotic) and moderate antifibrotic effects in preclinical animal experiments for liver diseases. Moreover, the effects of HB-EGF, HGF, and the combination of the two were examined to clarify the biologic roles and therapeutic potential of these hepatotrophic growth factors.

Materials and methods

Recombinant adenoviral vectors. Replication-defective recombinant adenoviral vectors (Ads) under the transcriptional control of cytomegalovirus immediate-early gene enhancer and the chicken β-actin promoter were generated and prepared as described previously (20,29,35,38-40). Vectors included Ad.LacZ, Ad.HB-EGF and Ad.HGF, which express β-galactosidase, human HB-EGF and human HGF, respectively.

Animal experiments and ethics statement. The protocol for mouse experiments is shown in Fig. 1A. On day 0, male 5-6-week-old C57BL/6J mice (Japan Charles River Co., Yokohama, Japan) were injected in the tail vein with $1x10^{11}$ particles of Ad.LacZ (day 3, n=9; day 14, n=15), Ad.HB-EGF (day 3, n=11; day 14, n=13), or Ad.HGF (n=10), or a combination of Ad.HB-EGF and Ad.HGF ($2x10^{11}$ total particles; n=10). The mice were then subjected to double ligation of the common bile duct essentially as described previously with minor modifications (5,41). Some animals were sacrificed by an overdose of diethyl ether anesthesia on day 3, whereas blood was collected from the tail veins of other animals on day 5 prior to sacrifice on day 14 (Fig. 1A). Liver and blood samples were collected at the time of sacrifice.

All animal studies were performed in accordance with the National Institutes of Health guidelines and with the approval of the Frontier Science Research Centre, Kagoshima University (permit number: MD09013). All efforts were made to minimize suffering.

Biochemical analysis. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T-Bil) were measured using an automated analyzer (SPOTCHEM EZ SP-4430; Arkray, Kyoto, Japan).

Histopathologic analysis. Formalin-fixed, paraffin-embedded liver tissue samples were stained with hematoxylin (Merck KGaA, Darmstadt, Germany) and eosin (Wako, Tokyo, Japan), or Masson's trichrome (Trichrome Stain (Masson) kit; Sigma-Aldrich, St Louis, MO, USA). For morphometric analysis of hepatocyte death, the areas containing biliary infarcts (clusters of characteristic oncotic hepatocytes) were assessed by counting 13,280 points on an image (x40, magnification; Powered BX-41; Olympus, Tokyo, Japan) as previously described with some modifications (42-45). To assess liver fibrosis quantitatively, areas stained blue with Masson's trichrome were measured using Image J software (National Institutes of Health). To assess *in vivo* adenoviral gene transduction efficiency, staining was performed using O-nitrophenyl-β-D-galactopyranoside (X-gal) and frozen liver tissues from the mice that received Ad.LacZ injections as described previously (20,35,44).

Immunohistochemistry was performed using antibodies specific for goat polyclonal anti-human HB-EGF (AF-259-NA; R&D systems, Minneapolis, MN, USA), rat monoclonal anti-mouse Ki-67 (M7249; Dako, Glostrup, Denmark) and rabbit polyclonal anti-mouse collagen IV (ab6586; Abcam, Cambridge, UK) as described previously (29,30,35). The immunofluorescent images were captured using a fluorescent

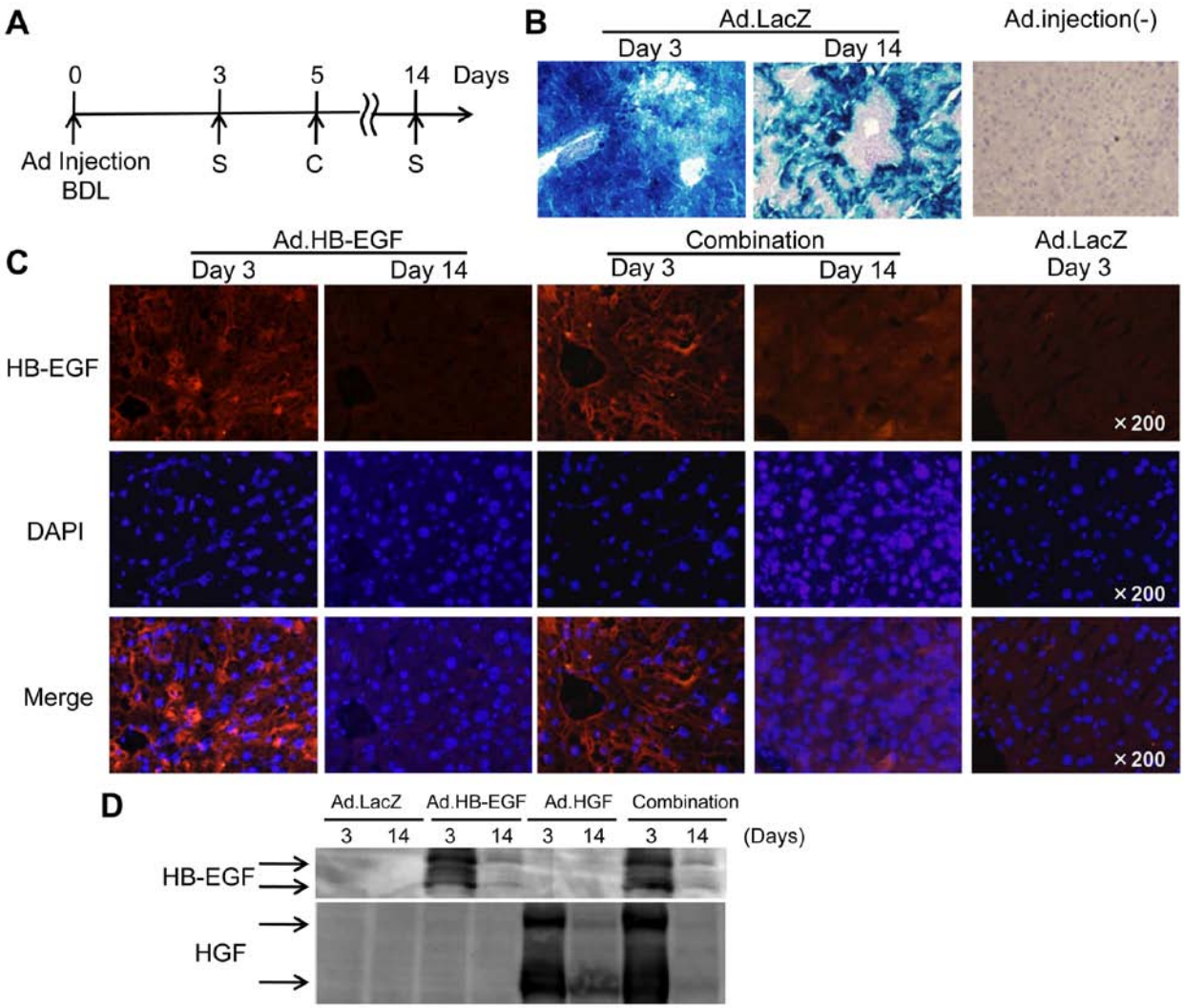

Figure 1. Adenoviral gene transduction and expression in mice subjected to BDL. (A) Experimental protocol for the following experiments. On day 0, mice received an intravenous injection of 1 x 10^{11} particles of either Ad.LacZ, Ad.HB-EGF, or Ad.HGF, or a combination of Ad.HB-EGF and Ad.HGF (2 x 10^{11} particles in total). Some animals were sacrificed (S) on day 3, whereas blood was collected (C) from other animals under anesthesia on day 5 prior to sacrifice on day 14. Liver and blood samples were collected at the time of sacrifice. (B) X-gal staining of liver samples, 3 and 14 days after Ad.LacZ injection and BDL (x200 magnification). (C) Immunohistochemical staining for human HB-EGF in liver samples 3 and 14 days after BDL and intravenous injection of control Ad.LacZ, Ad.HB-EGF, or a combination of Ad.HB-EGF and Ad.HGF (x200 magnification). (D) Western blot analysis for human HB-EGF and human HGF on days 3 and 14 after BDL and injection of control Ad.LacZ, Ad.HB-EGF, Ad.HGF, or a combination of Ad.HB-EGF and Ad.HGF. BDL, bile duct ligation. Ad, adenoviral vector; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor.

microscope (AxioObserver.A1; Carl Zeiss, Oberkochen, Germany.

To detect apoptotic cells, a terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay (ApopTag Kit; Chemicon International, Billerica, MA, USA) was performed according to the manufacturer's instructions.

TUNEL-positive or Ki-67-positive hepatocytes were counted and averaged in 30 randomly selected fields at x200, magnification (17,29). TUNEL-positive, Ki-67-positive, and all epithelial cells in the interlobular bile ducts were counted and the percentages of positive cells were calculated.

Western blot analysis. Liver tissue was homogenized in lysis buffer containing 15 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.15 M NaCl, 2 mM ethylenediaminetetraacetic acid (pH 8), 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, and Tris-Cl (pH 7.5). Western blot analysis was performed as described previously (17,30) using primary antibodies specific for goat polyclonal anti-human HB-EGF, goat polyclonal anti-human HGF (AB-294-NA; R&D Systems), mouse monoclonal anti-mouse α-smooth muscle actin (α-SMA; A2547; Sigma-Aldrich), rabbit polyclonal anti-mouse Bcl-2 (sc-492; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-mouse Bcl-xL (sc-634; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-mouse Bax (sc-7480; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-mouse collagen I (ab88147; Abcam) or mouse monoclonal anti-mouse α-tubulin (T6074; Sigma-Aldrich). The densities of the bands were measured, and the signal ratios of α-SMA/α-tubulin and collagen I/α-tubulin were calculated. For detection of each molecule, 25 μg of protein was loaded (40 μg in the case of α-SMA). Band densities were measured using ImageJ software.

Statistical analysis. Values are expressed as the means ± standard errors (SE). Differences between/among groups were evaluated using the unpaired Student's t-test for comparisons between two groups or one-way ANOVA for multiple-group comparisons (Statmate III software, ATMS Co., Ltd., Tokyo, Japan). P <0.05 was considered to indicate a statistically significant difference.

Results

Adenoviral gene transduction efficiency and expression in mice subjected to BDL. We first examined adenoviral gene transduction efficiency and the persistence of transgene expression in mouse livers after BDL (Fig. 1B). The intravenous injection of 1 x 10^{11} particles of Ad.LacZ resulted in close to 100% gene transduction among hepatocytes 3 days after Ad injection and BDL, whereas approximately half of the hepatocytes showed transgene expression on day 14 (Fig. 1B). On day 3, hepatocytes from mice treated with Ad.HB-EGF or the combination of Ad.HB-EGF and Ad.HGF immunohistochemically showed intense membrane staining of human HB-EGF (membrane-anchored proHB-EGF) and moderate staining intensity in the cytoplasm (likely endocytosed sHB-EGF) (Fig. 1C). The percentages of hepatocytes showing strong human HB-EGF expression and human HB-EGF-specific staining in individual hepatocytes were markedly lower on day 14. The high and low expression of human HB-EGF on days 3 and 14, respectively, was confirmed by western blot analysis (Fig. 1D). Similarly, western blot analysis demonstrated that the expression of human HGF was high on day 3 and low on day 14 after BDL and injection of Ad.HGF or the combination of Ad.HB-EGF and Ad.HGF (Fig. 1D); immunohistochemical confirmation of this result was hampered owing to the lack of a reliable anti-HGF antibody for immunohistochemistry. Taken together, the results showed efficient adenoviral gene transduction, robust expression of the transgenes (human HB-EGF and HGF), and limited periods of (i.e., several days) transgene expression in the BDL livers following intravenous Ad injection. These findings are consistent with those from previous studies, although the percentages of transduced hepatocytes and transgene expression levels vary based on the transgenes and transcriptional regulatory elements (i.e., promoters) in the Ads (29,44,46).

Liver enzyme levels after HB-EGF and/or HGF gene therapy in mice subjected to BDL. By carefully examining the time course of pathologic changes after BDL, previous studies demonstrated that hepatocyte injury and proliferation peaked at 3 and 5 days after BDL, respectively, and that fibrosis was fully established 14 days after BDL (5,41). Based on these results and the limited periods during which transgenes were expressed (Fig. 1), we selected 3, 5, and 14 days after BDL to experimentally represent the acute, subacute, and chronic phases, respectively.

In accordance with previous findings, AST and ALT levels peaked 3 days after BDL and the injection of control Ad.LacZ (Fig. 2); AST levels were 60 times higher than normal, whereas ALT levels increased 42 fold (1260±312 IU/l). The increases in AST and ALT levels were significantly attenuated in mice injected with Ad.HB-EGF (AST, 1586±309 IU/l and ALT, 517±93 IU/l; 2.6 and 2.4 times as low as those in the control Ad.LacZ-treated mice, respectively). By contrast, Ad.HGF injection mildly, but not to a statistically significantly degree, inhibited the elevations in AST and ALT levels on day 3. On days 5 and 14 after BDL in all treatment groups, AST and ALT levels returned to approximately 2000 IU/l and 1000 IU/l, respectively. On day 5, no significant differences in AST and ALT levels were observed among mice injected with control Ad.LacZ, Ad.HB-EGF, Ad.HGF or the combination of Ad.HB-EGF and Ad.HGF. On day 14, significant differences were observed between either Ad.HGF or the combination versus the control Ad.LacZ in AST levels and between Ad.HGF and the combination in ALT levels.

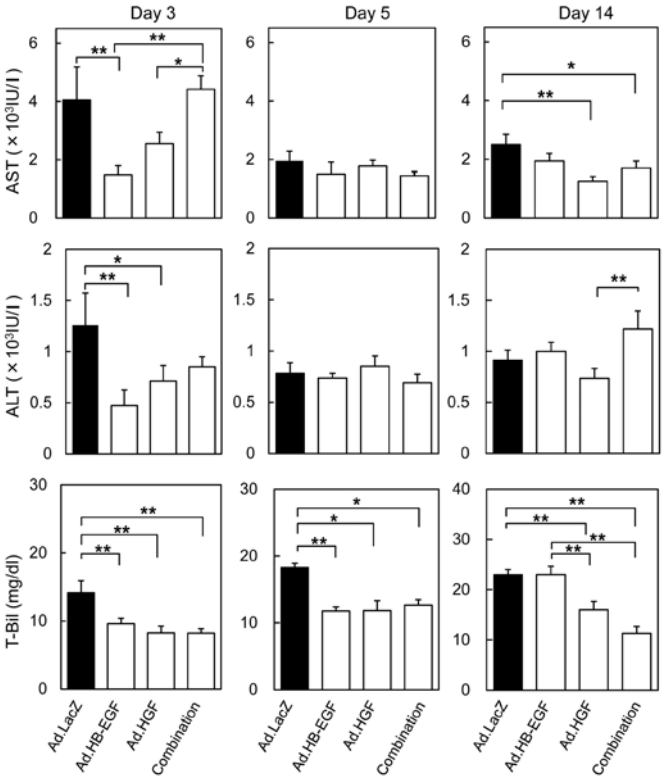

Figure 2. Serum biochemical analysis following Ad injection and BDL. Blood was collected on days 3, 5, and 14 (see Fig. 1A), and serum AST, ALT and T-Bil levels were assessed. All data are expressed as the means ± SE (*P<0.05, **P<0.01 Ad.HB-EGF, Ad.HGF, a combination of Ad.HB-EGF and Ad.HGF and Ad.LacZ each). Ad, adenoviral vector; BDL, bile duct ligation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor.

On the other hand, serum T-Bil levels in mice treated with control Ad.LacZ progressively increased to 14.1±1.7 mg/dl, 18.3±1.4 mg/dl and 23.0±1.8 mg/dl (108, 141 and 177 times higher than normal levels) on days 3, 5 and 14 after BDL, respectively (Fig. 2), suggesting that BDL-induced cholestasis worsened over time. The increases in serum T-Bil levels were significantly attenuated on days 3 and 5 by injection of Ad.HB-EGF, Ad.HGF or both Ad.HB-EGF and Ad.HGF. The further increases in T-Bil observed on day 14 were inhibited by injection of Ad.HGF alone or Ad.HB-EGF and Ad.HGF.

Thus, HB-EGF alone produced potent cytoprotective effects during acute hepatocyte injury after BDL, and the anticholestatic effects of HGF were stronger than those of HB-EGF during the chronic stage. These findings suggest that,

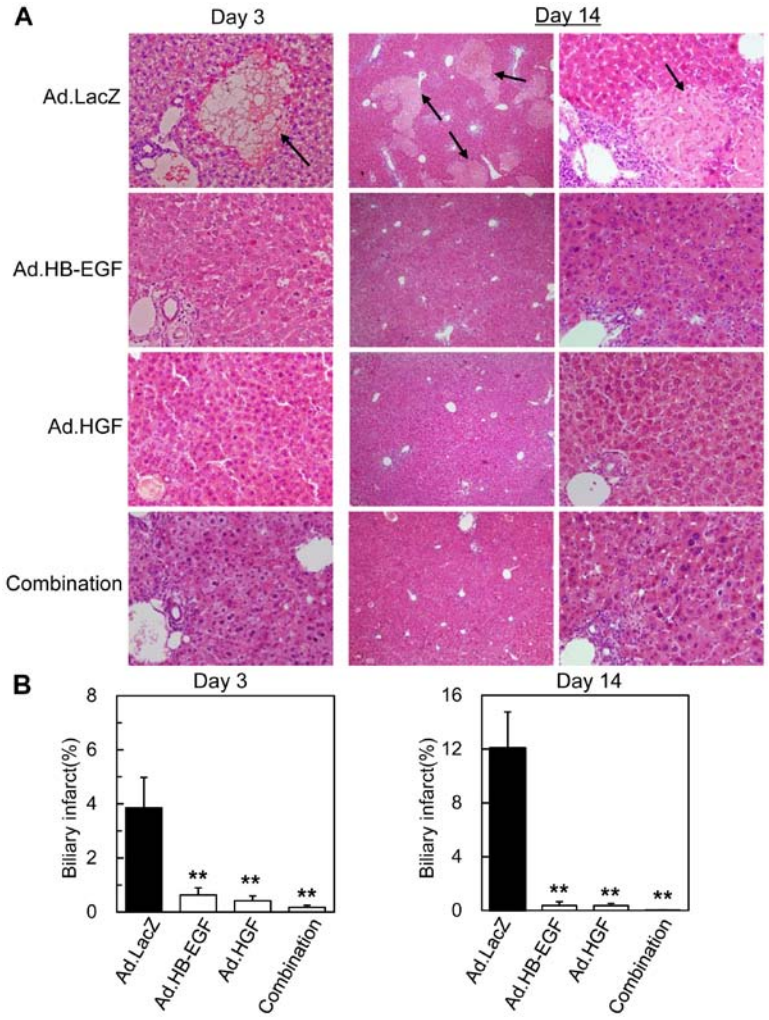

Figure 3. Liver histopathology after BDL and gene therapy with HB-EGF and/or HGF. (A) Liver sections obtained from mice 3 days (left panels, x200 magnification) or 14 days (middle and right panels, x40 and x200, respectively) after BDL and injection of Ad.LacZ, Ad.HB-EGF, Ad.HGF, or the combination of Ad.HB-EGF and Ad.HGF were stained with hematoxylin and eosin. Black arrows indicate typical biliary infarcts. (B) Morphometric and quantitative analyses of biliary infarcts on days 3 and 14. The percentages of infarcted areas among the parenchymal areas were calculated. All data are expressed as the means ± SE (**P<0.01, Ad.HB-EGF, Ad.HGF, or a combination to the two vs. control Ad.LacZ). No significant differences were observed among the Ad.HB-EGF-, Ad.HGF-, and combination-treated samples. BDL, bile duct ligation; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor; Ad, adenoviral vector.

following BDL, HB-EGF and HGF are therapeutic predominantly during the acute and chronic phases, respectively.

Gene therapy with HB-EGF and/or HGF potently attenuates histologic liver injuries and hepatocyte oncosis (necrosis). Previous studies demonstrated that the characteristic histopathologic feature in BDL-induced liver injury is 'biliary infarct', defined as clusters of oncotic hepatocytes; oncosis is a characteristic type of liver cell necrosis with cytoplasmic swelling, disruption of plasma membrane integrity and decreased nuclear staining in response to obstructive cholestasis. Similar to previous studies (5-7), histopathologic and morphometric analyses of livers 3 days after BDL in mice treated with control Ad.LacZ revealed severe liver injuries with prominent biliary infarcts (3.9±1.2%; percentage of infarcted areas in the parenchymal areas) - defined as clusters of swollen hepatocytes lacking nuclear staining (oncosis) - in the periportal and/or the midzonal areas of the liver parenchyma (Fig. 3). In actuality, the hepatocytes showing the typical morphology of oncosis were TUNEL-negative on day 3 (Fig. 4A). Larger areas were affected by the biliary infarcts (12.1±2.7%) on day 14 (Fig. 3), when the elevated serum AST and ALT levels had somewhat abated, whereas serum T-Bil levels had increased (Fig. 2), suggesting that cholestatic liver injuries worsened after BDL.

By contrast, 3 and 14 days after BDL, minimal histopathologic findings were observed in the livers of mice treated with Ad.HB-EGF, Ad.HGF, or both Ads, including few biliary infarcts (on day 3, 0.6±0.3%, 0.4±0.2%, and 0.2±0.1%, respectively; on day 14, 0.4±0.3%, 0.4±0.2% and 0.04±0.02%, respectively). Thus, HB-EGF and HGF potently inhibited BDL-induced liver injury based on histopathologic analyses, which showed effects that were greater than those observed using serum levels of liver enzymes; the same tendency was observed in previous studies that examined these growth factors in the treatment of acute liver injuries (17,18,29,30). More importantly, the data suggest that HB-EGF and HGF are potently antioncotic (antinecrotic) for hepatocytes.

Antiapoptotic effects of gene therapy with HB-EGF and/or HGF in BDL-induced liver injury. To examine whether

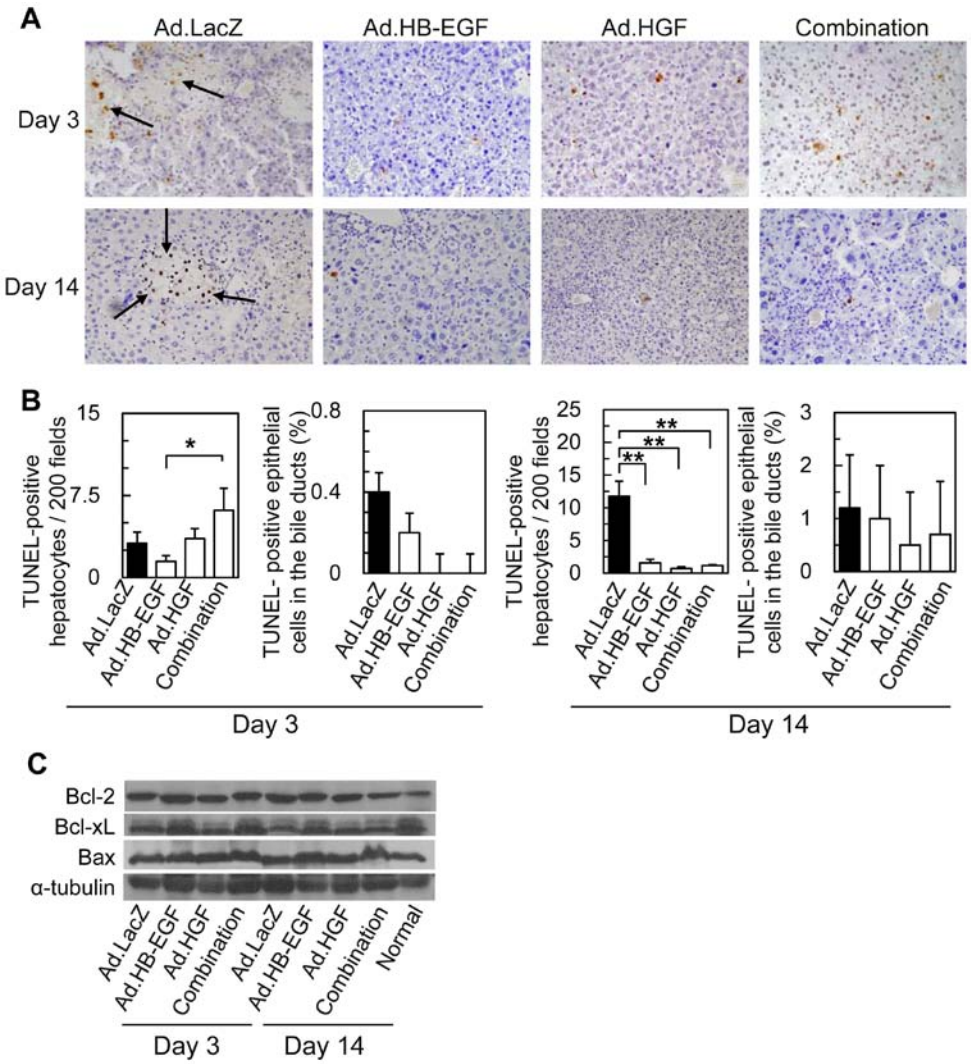

Figure 4. TUNEL staining of liver samples and western blot analysis of Bcl/Bax-family proteins in livers after Ad injection and BDL. (A) TUNEL staining of livers 3 and 14 days after BDL and injection of Ad.LacZ, Ad.HB-EGF, Ad.HGF, or the combination of Ad.HB-EGF and Ad.HGF (x200 magnification). The black arrows indicate TUNEL-positive cells. (B) Morphometric and quantitative analyses of TUNEL-positive hepatocytes and epithelial cells of the interlobular bile ducts. All data are expressed as the means ± SE (*P<0.05, **P<0.01 Ad.HB-EGF, Ad.HGF, a combination of Ad.HB-EGF and Ad.HGF and Ad.LacZ each). No significant differences in the numbers of TUNEL-positive hepatocytes or epithelial cells in bile ducts were noted among samples treated with Ad.HB-EGF, Ad.HGF, or the combination of Ad.HB-EGF and Ad.HGF. (C) Western blot analysis of Bcl-2, Bcl-xL, Bax, and α-tubulin (control) in mouse liver samples 3 and 14 days after BDL and Ad injection. TUNEL, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick-end labeling; Ad, adenoviral vector; BDL, bile duct ligation; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor.

HB-EGF and HGF prevent BDL-induced apoptosis of hepatocytes, apoptotic cells were detected using *in situ* TUNEL assays (Fig. 4A) and morphometrically analyzed (Fig. 4B). Previous studies demonstrated that hepatocytes primarily die during acute cholestasis via oncosis, although the apoptosis of hepatocytes is also involved in BDL-induced liver injury (5,7-9). Correspondingly, the majority of hepatocytes in biliary infarcts from control Ad.LacZ-treated mice were TUNEL-negative, and few hepatocytes (3.2±1.0 cells in 200 fields) around the periphery of the biliary infarcts were TUNEL-positive 3 days after BDL (Fig. 4A and B). On day 14, the number of TUNEL-positive hepatocytes increased to 3.7 times as many as that observed on day 3 (Fig. 4B).

Ad.HB-EGF slightly reduced the number of TUNEL-positive hepatocytes 3 days after BDL, although no statistically significant difference was noted among the groups (Fig. 4B). Fourteen days after BDL, significantly (around 10-fold) fewer TUNEL-positive hepatocytes were detected in the livers of mice treated with Ad.HB-EGF, Ad.HGF, or a combination of Ad.HB-EGF and Ad.HGF compared with Ad.LacZ-treated mice; differences among the three treatment groups were not statistically significant.

On the other hand, in the mice treated with control Ad.LacZ, <1% of epithelial cells in the interlobular bile ducts were TUNEL-positive 3 days after BDL, with a slight increase to 1.2% after 14 days (Fig. 4B). The treatments appeared to slightly reduce the percentage of TUNEL-positive epithelial cells in the bile ducts, although no statistically significant difference was noted among the groups.

These data clearly indicate that HB-EGF and HGF exert antiapoptotic and antioncotic effects on hepatocytes, even though the expression of representative Bcl-2/Bax-family proteins - Bcl-2, Bcl-xL and Bax - were not markedly affected by any of the treatment approaches (Fig. 4C).

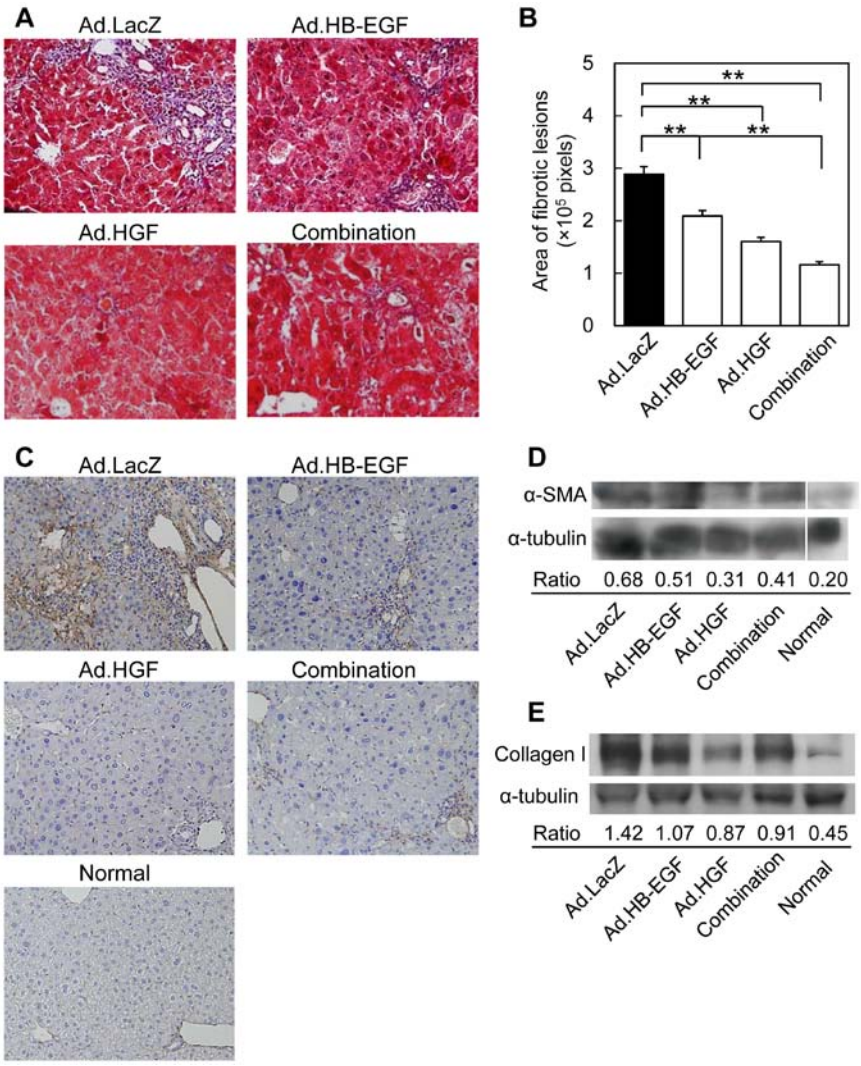

Figure 5. Liver fibrosis 14 days after Ad injection and BDL. (A). Representative histologic images of liver samples stained with Masson's trichrome (x200 magnification) and (B) morphometric and quantitative analyses of the stained fibrotic areas. All data are expressed as the means ± SE. (**P<0.01 Ad.HB-EGF, Ad.HGF, a combination of Ad.HB-EGF and Ad.HGF and Ad.LacZ). (C) Immunohistochemical staining of collagen-IV (x200 magnification). (D and E). Western blot analysis of (D) α-SMA or (E) collagen I and α-tubulin (control). Signal ratios of α-SMA/α-tubulin and collagen I/α-tubulin are shown below the bands in each lane. Ad, adenoviral vector; BDL, bile duct ligation; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor; α-SMA, α-smooth muscle actin.

More potent inhibitory effects of HGF than HB-EGF against liver fibrosis. To examine the inhibitory effects of HB-EGF and HGF on liver fibrosis, collagen fibers in the livers of mice 14 days after BDL were stained with Masson's trichrome, and the fibrous areas were quantified. In accordance with previous findings (5), Ad.LacZ-treated control mice showed severe fibrosis, including bridging by connective tissue that linked different portal areas (Fig. 5A & B). Ad.HB-EGF treatment resulted in significantly smaller fibrous areas with fainter and thinner fibrous tissues. On the other hand, treatment with either Ad.HGF or the combination of Ad.HB-EGF and Ad.HGF resulted in larger decreases in the areas affected by fibrous tissues. This result was supported by immunohistochemical staining of collagen IV, which forms a basement membrane-like structure in the space of Disse (Fig. 5C) (47).

To analyze activated hepatic stellate cells (5,48), α-SMA protein levels were examined by western blot analysis of liver samples obtained on day 14 (Fig. 5D). The expression of α-SMA increased following BDL in Ad.LacZ-treated mice compared with that in normal mice. Although α-SMA protein levels were slightly lower in Ad.HB-EGF-treated mice, they were markedly attenuated by Ad.HGF and the combination of Ad.HGF and Ad.HB-EGF. The same tendency was noted for collagen I protein expression, increases of which are a cardinal feature of liver fibrosis with elevated deposition observed as hepatic stellate cells are activated to become α-SMA-positive myofibroblasts (49). Taken together, these results indicate that, compared with HB-EGF, HGF more potently inhibits liver fibrosis.

HB-EGF and HGF additively induce liver regeneration. We assessed the effects of HB-EGF, HGF and a combination of the two growth factors on liver regeneration following BDL by examining the number of Ki-67-positive cells (Fig. 6). The numbers of Ki-67-positive, regenerating hepatocytes 3 days after BDL significantly increased by 2.1-, 2.4-, and 3.4-fold following treatment with Ad.HB-EGF, Ad.HGF, and the combination, respectively, compared with control Ad.LacZ; no significant differences were noted among the three

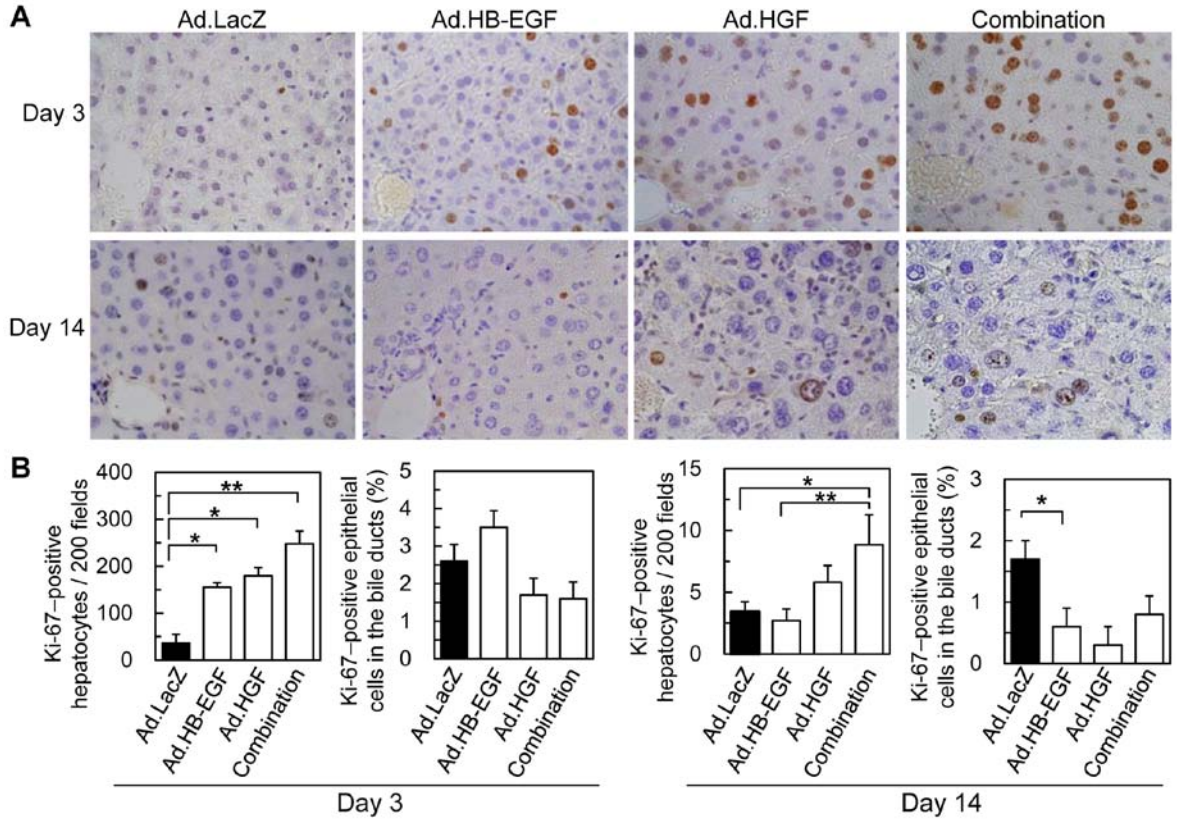

Figure 6. Immunohistochemical staining of Ki-67 in livers following BDL and Ad injections. (A) Representative images of Ki-67 staining 3 and 14 days following BDL and injection of Ad.LacZ, Ad.HB-EGF, Ad.HGF, or a combination of Ad.HB-EGF and Ad.HGF. (B) Morphometric and quantitative analyses of Ki67-positive cells. All data are expressed as the means ± SE (*P<0.05, **P<0.01 Ad.HB-EGF, Ad.HGF, a combination of Ad.HB-EGF and Ad.HGF and Ad.LacZ). BDL, bile duct ligation; Ad, adenoviral vector; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor.

treatment groups. On the other hand, the number of Ki-67-positive hepatocytes on day 14 in control Ad.LacZ-treated mice was reduced by >90% in comparison with the results observed on day 3. Although no significant differences were noted among the groups treated with Ad.LacZ, Ad.HB-EGF, or Ad.HGF, the combination of Ad.HB-EGF and Ad.HGF significantly (2.6-fold) enhanced liver regeneration during the chronic phase after BDL compared with control Ad.LacZ. By contrast, no significant differences were noted among the groups in the percentages of Ki-67-positive epithelial cells in the interlobular bile ducts on day 3 or 14. These results suggest that HB-EGF and HGF preferentially induce hepatocyte regeneration after BDL, and that combination therapy additively increases and sustains the regenerative activities of these growth factors.

Discussion

In this study, we, for the first time to the best of our knowledge, examined the therapeutic effects of HB-EGF in cholestatic liver injuries, and compared the benefits to those associated with HGF. Notably, the protective effects of HB-EGF against acute liver injury - assessed based on serum transaminase levels - were greater than those of HGF, which is consistent with previous findings regarding Fas-induced acute liver injury (29). Unlike HGF, however, the longer term anticholestatic and antifibrotic effects of HB-EGF after BDL were moderate. These differences may reflect differences in the biologic functions of these growth factors in the liver, an idea that is supported by the different phenotypes observed in HGF and HB-EGF null mice (50-52). Our results also suggest that there is a time period during which the effects of the growth factors for liver injury are most pronounced. In fact, a previous study has shown that endogenous HB-EGF mRNA levels increase more rapidly than those of endogenous HGF mRNA during liver regeneration after partial hepatectomy (53). In addition, we have revealed that HB-EGF gene therapy is more protective and mitogenic than HGF gene therapy for Fas-induced acute liver injury (29). Taken together, our data suggest that HB-EGF and HGF have overlapping functions, including antiapoptotic, antioncotic (antinecrotic), anticholestatic and mitogenic activities in hepatocytes, whereas these growth factors predominantly are acutely cytoprotective and chronically antifibrotic, respectively.

In response to BDL-induced cholestatic liver injury, the primary target cells for these growth factors may be hepatocytes, because the cytoprotective and regenerative activities were prominent in hepatocytes, whereas effects in the interlobular bile ducts were not apparent. Taken together with the results of previous studies (17,18,29,30), the results of this study suggest that HGF and HB-EGF may protect hepatocytes against a range of hepatotoxic stimuli, including the bile salt-induced toxicity applied in this model (5,6). Other anticholestatic mechanisms, including upregulation of basolateral bile acid/bilirubin transporters and kidney excretion of bile salts/bilirubin, should be carefully examined in future studies.

Furthermore, this study revealed, for the first time to the best of our knowledge, that HB-EGF exerts potent antioncotic (antinecrotic) effects on hepatocytes *in vivo*. Notably, the expression of Bcl/Bax-family proteins, which were altered by HGF or HB-EGF in previous studies to reduce Fas-induced hepatocyte apoptosis (17,18,29,30), were not markedly affected in this study. This likely reflects a smaller role for apoptosis relative to oncosis in BDL-induced cholestatic liver injury, and suggests that different cytoprotective mechanisms are responsible for antiapoptotic and antioncotic (antinecrotic) effects. However, further elucidation of the underlying molecular mechanism is currently hampered by the paucity of overall molecular information regarding oncotic processes.

As mentioned earlier, previous studies have yielded controversial results regarding whether HB-EGF inhibits or stimulates liver fibrosis *in vivo*, as well as the relative antifibrotic effect of HB-EGF in comparison to that of HGF (32-35). In this study, we showed that HB-EGF exerts moderate but significant antifibrotic effects. Sequential events that result in liver fibrosis have been previously reported (5,49); during hepatic injury, hepatic stellate cells are activated to become α-SMA-positive myofibroblasts and produce collagen matrix, including predominantly collagen I (5,49). Notably, the examination of areas of liver fibrosis, deposition of collagen IV (another collagen associated with liver fibrosis), and α-SMA and collagen I protein expression demonstrated that HB-EGF and HGF exerted moderate and strong antifibrotic effects, respectively. The moderate but significant antifibrotic effects of HB-EGF may be clinically meaningful because the induction of fibrosis by a cytoprotective agent may diminish its therapeutic utility for chronic liver diseases.

Clinically, these results suggest that HB-EGF or HGF may be effective for cholestatic liver injury. Moreover, combination therapy may be more beneficial based on the greater anticholestatic and regenerative activities observed in hepatocytes during the chronic phase. This study used an *in vivo* adenoviral gene transduction strategy to investigate whether HB-EGF has the potential to be used as a therapeutic agent for diverse hepatic disorders, particularly those with complex pathogenesis, including hepatocyte necrosis, liver fibrosis, and cholestatic liver injuries, as well as the differences in the therapeutic actions and pathophysiologic roles of HB-EGF and HGF (20,29,35,40,54,55). Future preclinical studies should examine long-term effectiveness and optimize pharmacotherapeutic protocols for each liver disease, including the timing of growth factor administration, the effects of gene vs. protein therapy, clinically appropriate vectors, and any treatment-related adverse effects, including the potential for hepatocarcinogenesis. Nevertheless, the dearth of pharmacologic agents that effectively inhibit cholestatic liver injuries suggests that HB-EGF and/or HGF should be developed as clinical therapies.

In summary, this study revealed that HB-EGF as well as HGF inhibited BDL-induced cholestatic liver injury by predominantly exerting acute cytoprotective and chronic antifibrotic effects, respectively. Moreover, combining the growth factors enhanced the anticholestatic effects and liver regeneration during the chronic phase. These results provide a foundation for novel pharmacotherapeutic approaches using HB-EGF and HGF, and aid in elucidating the pathophysiologic roles of these hepatotrophic growth factors.

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