metalloproteinases through the upregulation of tissue inhibitors of metalloproteinases in activated HSCs.

In order to elucidate the mechanisms of fibrogenesis and to evaluate the anti-fibrogenic effects of various agents used to treat liver cirrhosis, considerable efforts have been made to create appropriate animal models, in which liver fibrosis is chemically or genetically induced [6–9]. The cell strain M was established by Katsuta and Takaoka from normal rat liver cells, and transformed by Nagisa culture and treatment with 4-dimethyl-aminoazobenzene [10]. M cells are characterized by the active formation of a collagen fiber network: thin, fiber-like structures appear, and increase with time, resulting in the formation of a network of thick collagen fibers, which were identified by silver impregnation [11,12]. Because the features of collagen fibers surrounding bundles of M cells resemble the histological findings of hepatic fibrosis, this culture system has been used as an in vitro model of hepatic fibrosis to evaluate the ability of agents to suppress fibrogenesis [13,14].

In the present study, to clarify the features of M cells, we examined expression of genes specific for hepatocytes, biliary epithelial cells or HSCs using immunohistochemistry, and evaluated the effect of treatment with TGF-β1 on the formation of a collagen fiber network. Additionally, we examined the anti-fibrogenic effects of taurine, a sulfur-containing β-amino acid with antioxidant properties [15], using this in vitro model of hepatic fibrosis.

2. Materials and methods

2.1. Cell culture

The rat liver epithelial cell line, M, was kindly provided from Dr. Takaoka (Memorial Institute of Tissue Culture, Utsunomiya, Japan). M cells were cultured in DM-201 medium [16] supplemented with ascorbic acid (final concentration 1 mM) and 5% calf serum. The cells were plated at 1×10^5 cells/well in 4-well chamber slides (Nalge Nunc International K.K., Rochester, NY, USA), and cultured in the presence or absence of TGF-β1 (1 ng/ml) or various concentrations of taurine (10–50 mM). After 6-day culture, the cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and were subjected to silver impregnation.

The growth of M cells was determined by colorimetric quantitation of the viable cell number. M cells (3×10^3/well) were plated in 96-well multiplates. One day later, the cells were treated with TGF-β1 (1 ng/ml) for 48 h. To quantify cell viability, the medium was replaced with 0.1 ml of fresh medium, and 10 μl of TetraColor ONE (SEIKAGAKU, Tokyo, Japan) was added to each well. After incubation for 1 h, the color reaction was quantitated using an automatic plate reader (Well-reader, Seikagaku) at 450 nm with a reference filter of 630 nm.

2.2. Immunohistochemistry of M cells

The cells were fixed with 10% formaldehyde for 30 min at room temperature. For immunohistochemistry, the slides were treated with 3% H_2O_2 in PBS for 15 min, and washed twice, followed by blocking in 3% bovine serum albumin in PBS for 1 h at room temperature. The sections were then incubated with the following primary antibodies 16 h at 4 °C: anti-CK8 (PROGEN Biotechnik GmbH, Heidelberg, Germany), anti-CK18 (PROGEN Biotechnik GmbH), anti-CK19 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), anti-α-smooth muscle actin (αSMA) (SIGMA, Saint Louis, Missouri, USA), anti-vimentin (NICHIREI, Tokyo, Japan) and anti-desmin (DAKO, Carpinteria, CA, USA). After washing, the sections were incubated with Envinson-labeled polymer reagent (DAKO) for 45 min at 37 °C. The reaction was detected by ImmunoPure metal enhanced diaminobenzidine tetrahydrochloride (DAB) substrate kit (Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin.

2.3. Reverse transcription (RT)-PCR

Total RNA was extracted from M cells by the acid guanidinium thiocyanate:phenol:chloroform method. To examine the expression of TGF-β1, -β2, TGF-β type I and II receptors, RT-PCR was performed using a set of primers listed in Table 1 for 28 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s after initial denaturation (94 °C for 5 min). The resultant fragments of TGF-β1, -β2, TGF-β type I and II receptors were 302 bp, corresponding to bases 878–1179 of rat TGF-β1 cDNA (GeneBank/European Molecular Biology Laboratory Data Bank accession no. NM021578) [17], 305 bp, bases 1136–1440 of rat TGF-β2 cDNA (NM031131) [18], 301 bp, bases 650–950 of rat TGF-β type I receptor cDNA (NM012775) [19], and 303 bp, bases 935–1237 of rat TGF-β type II receptor cDNA (NM031132) [20] in size, respectively.

2.4. Northern blot analysis

Total RNA was extracted from M cells treated with or without TGF-β1 (1 ng/ml) or taurine (10–50 mM) for 6

Table 1
Primers used

Primer name	Primers
TGF-β1	S: 5′-CGCCTGCAGAGATTCAAGTCA-3′ A: 5′-GGTCGGTTCATGTCATGGATG-3′
TGF-β2	S: 5′-AGATTCGCAGGTATCGATGGC-3′ A: 5′-CATGCCCCAGCACAGAAGTTA-3′
TGF-β receptor type I	S: 5′-TGCTACAAGAAAGCATCGGCA-3′ A: 5′-GCGAGTTTGATCATTCCTTCCA-3′
TGF-β receptor type II	S: 5′-CTCTACGTGCGCCAACAACAT-3′ A: 5′-GCATGAAACGCCGTGATCA-3′

days as described above. For detection of procollagen α1(I) mRNA expression, a cDNA clone Hf-677 encoding human procollagen α1(I) was used as a probe [21].

3. Results

3.1. M cells stain positive for CK8, CK18, vimentin and αSMA, but negative for CK19 and desmin

M cells were derived from rat liver epithelial cells and transformed to produce collagen fibers extensively in culture [11,12]. Therefore, we first examined the expression of markers for hepatocytes, biliary epithelial cells or HSCs. Although albumin production in the culture supernatants or its expression in M cells was not detected, expression of CK8 and CK18, both of which are markers of hepatocytes [22], was immunohistochemically observed (Fig. 1A and B). In contrast, M cells did not express CK19, a specific marker for biliary epithelial cells (Fig. 1C). Vimentin, a marker for liver epithelial cells or HSCs, and αSMA, which has been shown to be expressed specifically in activated HSCs, were also expressed, but expression of desmin, another marker for HSCs, was not detected (Fig. 1D, E and F). These results indicate the possibility that M cells exhibit the features of not only hepatocytes but also myofibroblasts, leading to their ability to produce a dense collagen fiber network.

3.2. TGF-β1 stimulates the formation of a collagen fiber network and expression of procollagen α1(I) mRNA

TGF-β is known to play a central role in liver fibrogenesis [4,5]. To clarify whether TGF-β is involved in collagen fiber network formation in M cells, we examined the expression of TGF-β and its receptors by RT-PCR (Fig. 2A). M cells expressed all transcripts for TGF-β1, -β2, TGF-β type I and II receptors, and TGF-β1 (~1.0 ng/ml) in the culture supernatant was also detectable by ELISA. Next, we examined

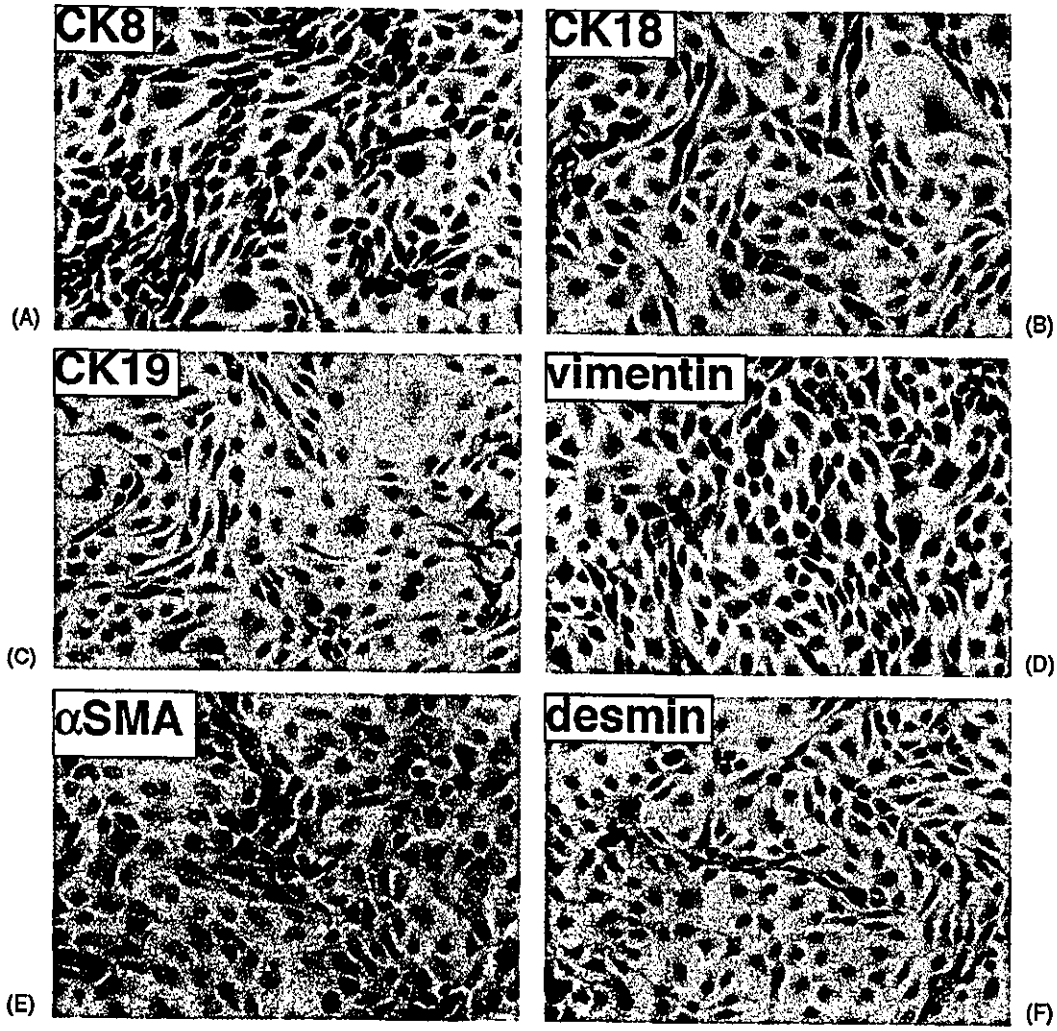

Fig. 1. M cells stained positive for CK8, CK18, vimentin and αSMA, but negative for CK19 and desmin. Expression of a sort of intermediate filaments was examined using immunohistochemistry as described in Section 2. M cells expressed CK8, CK18, vimentin and αSMA, but not CK19 and desmin (magnification 400×).

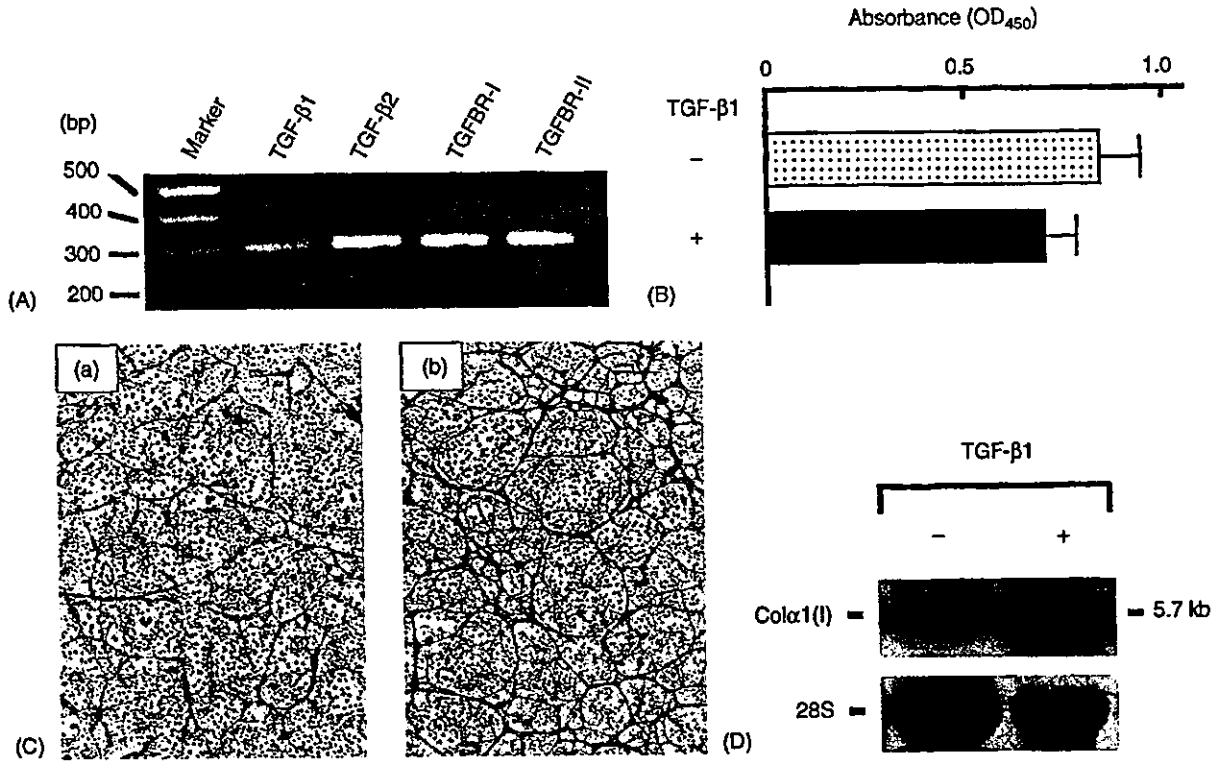

Fig. 2. TGF-β1 treatment stimulated collagen fiber formation and expression of procollagen α1(I) mRNA in M cells. (A) Expression of TGF-β1, -β2, TGF-β type I and type II receptors was examined by RT-PCR as described in Section 2. M cells expressed transcripts for TGF-β1, -β2, TGF-β type I and type II receptors. (B) M cells were incubated with or without TGF-β1 (1 ng/ml) for 48 h, and cell growth was determined by colorimetric quantitation of the viable cell number. Treatment with TGF-β1 slightly inhibited the prolifefration of M cells, but these differences were not statistical significant ($P = 0.06$). (C) M cells were cultured in the presence (b) or absence (a) of TGF-β1 (1 ng/ml) for 6 days, and subjected to silver impregnation. Formation of the collagen fiber network was markedly stimulated by treatment with TGF-β1 (magnification 100×). (D) Total RNA (10 μg) was isolated from M cells treated with or without TGF-β1, and Northern blotting was performed as described in Section 2. Treatment with TGF-β1 up-regulated the expression of procollagen α1(I) mRNA.

the effect of TGF-β1 treatment on the growth of M cells and the formation of a collagen fiber network (Fig. 2B and C). Treatment with TGF-β1 slightly inhibited the proliferation of M cells, but there was no statistically significant difference ($P = 0.06$) (Fig. 2B). When M cells were treated with 1 ng/ml of TGF-β1 for 6 days, collagen fiber network formation was significantly stimulated, and expression of procollagen α1(I) mRNA was also upregulated. These results indicate that TGF-β1 may function through autocrine and paracrine mechanisms, and TGF-β-mediated signaling pathways play an important role in the production of collagen fibers in M cells.

3.3. Treatment with taurine inhibits collagen fiber network formation and suppresses procollagen α1(I) expression in M cells

Several studies have reported that taurine is a potent anti-fibrogenic agent in the treatment of pulmonary fibrosis and renal sclerosis [23–25]. Therefore, we evaluated the effects of taurine on the formation of the collagen fiber network using M cells in culture. Although treatment with 10–50 mM of taurine did not affect either production in the culture supernatant or mRNA expression of TGF-β1 as well as the growth of M cells (data not shown), the formation of the collagen fiber network and expression of procollagen α1(I) mRNA were inhibited by taurine treatment in a dose dependent manner (Fig. 3A and B). Even in the presence of TGF-β1, treatment with taurine effectively reduced collagen fiber network formation and suppressed procollagen α1(I) expression (Fig. 4A and B). These results suggest that taurine may be a potential therapeutic agent against liver cirrhosis, and also indicate that this culture system is an appropriate in vitro model of hepatic fibrosis for the evaluation of the effects of potent anti-fibrogenic agents.

4. Discussion

The cell strain M was derived from normal rat liver cells, and is characterized by the active formation of collagen fibers [12]. In the present study, although M cells were morphologically epithelial-like and immunohistochemically positive for CK8 and CK18, expression of vimentin and αSMA, but not desmin, was observed. Also, morphologically M cells do not have either processes or

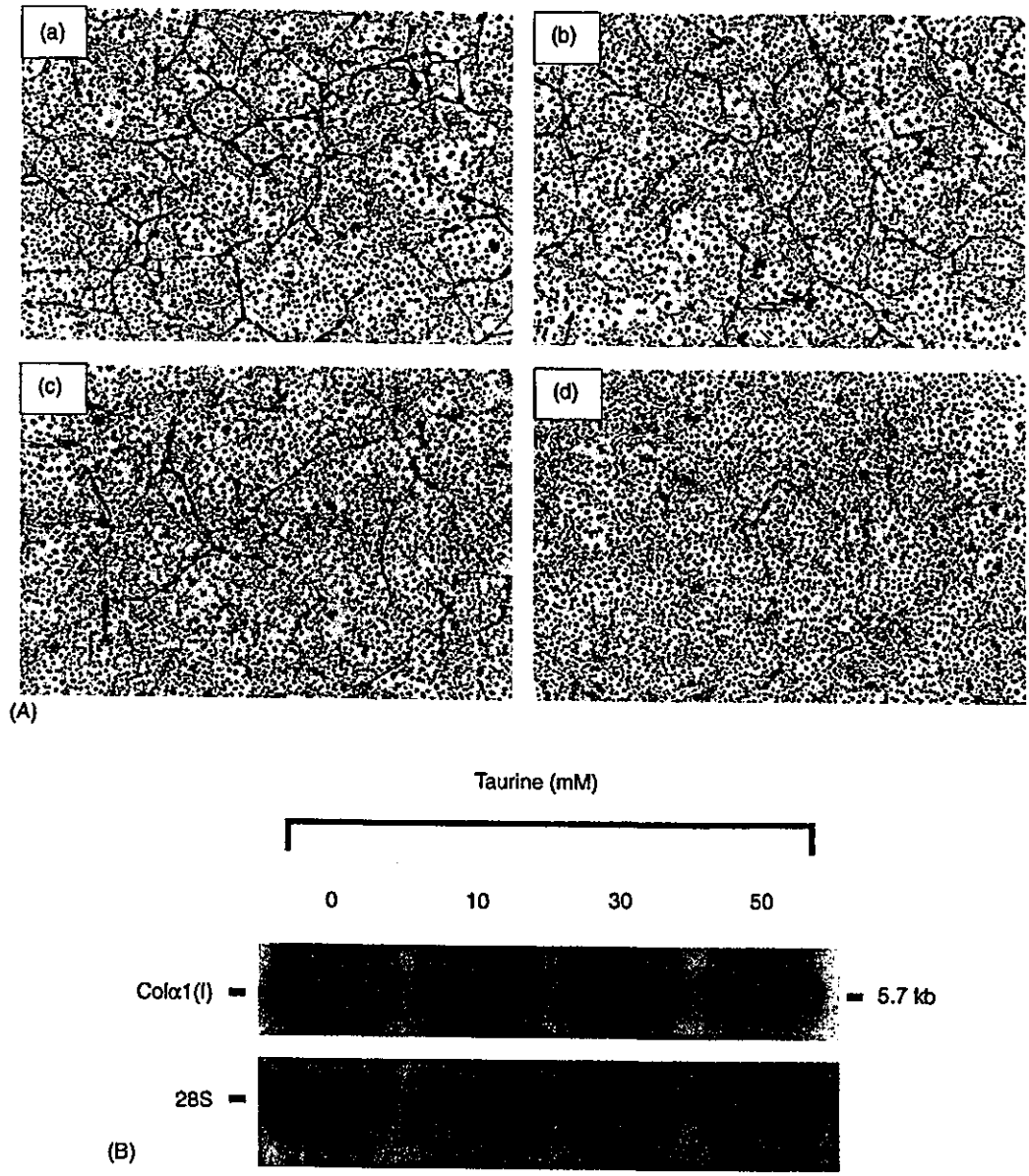

Fig. 3. Taurine significantly reduced the formation of a collagen fiber network, and suppressed procollagen α1(I) expression. (A) M cells were treated with 0 mM (a), 10 mM (b), 30 mM (c), and 50 mM (d) of taurine in the absence of TGF-β1, and subjected to silver impregnation. Treatment with taurine for 6 days suppressed the formation of the collagen fiber network in a dose dependent manner (magnification 100×). (B) Total RNA (10 μg) was extracted from M cells treated with various concentrations of taurine in the absence of TGF-β1, and Northern blot analysis was performed as described in Section 2. Treatment with taurine down-regulated expression of procollagen α1(I) mRNA.

lipid droplets, characteristic features of HSCs, indicating that M cells exhibit features of both liver epithelial cells and myofibroblasts. Recently, Valdes et al. have reported that a subpopulation of fetal rat hepatocytes, which survived TGF-β-mediated apoptosis, underwent an epithelial mesenchymal transition, resulting in the acquisition of vimentin expression and the lack of CK18 and E-cadherin [26]. Conversely, HSCs are known to undergo apoptosis after activation in culture [27], and recent investigations have reported the identification of desmin-negative HSCs as well as morphologically and functionally unique fibroblastic populations, which are positive for vimentin, but not for desmin [28,29]. Therefore, despite some features of epithelial cells, M cells might acquire the ability to produce collagen fibers like myofibroblasts during transformation or prolonged subcultivation.

TGF-β is widely regarded as pro-fibrogenic in liver injury, and this cytokine stimulates ECM production not only by HSC but also by sinusoidal endothelial cells [5]. In the present study, M cells expressed TGF-β1, -β2, TGF type I and II receptors, and TGF-β1 (~1 ng/ml) were detected in the culture supernatant. Additionally, the formation of a collagen fiber network in M cells and the expression of procollagen α1(I) mRNA were stimulated by treatment

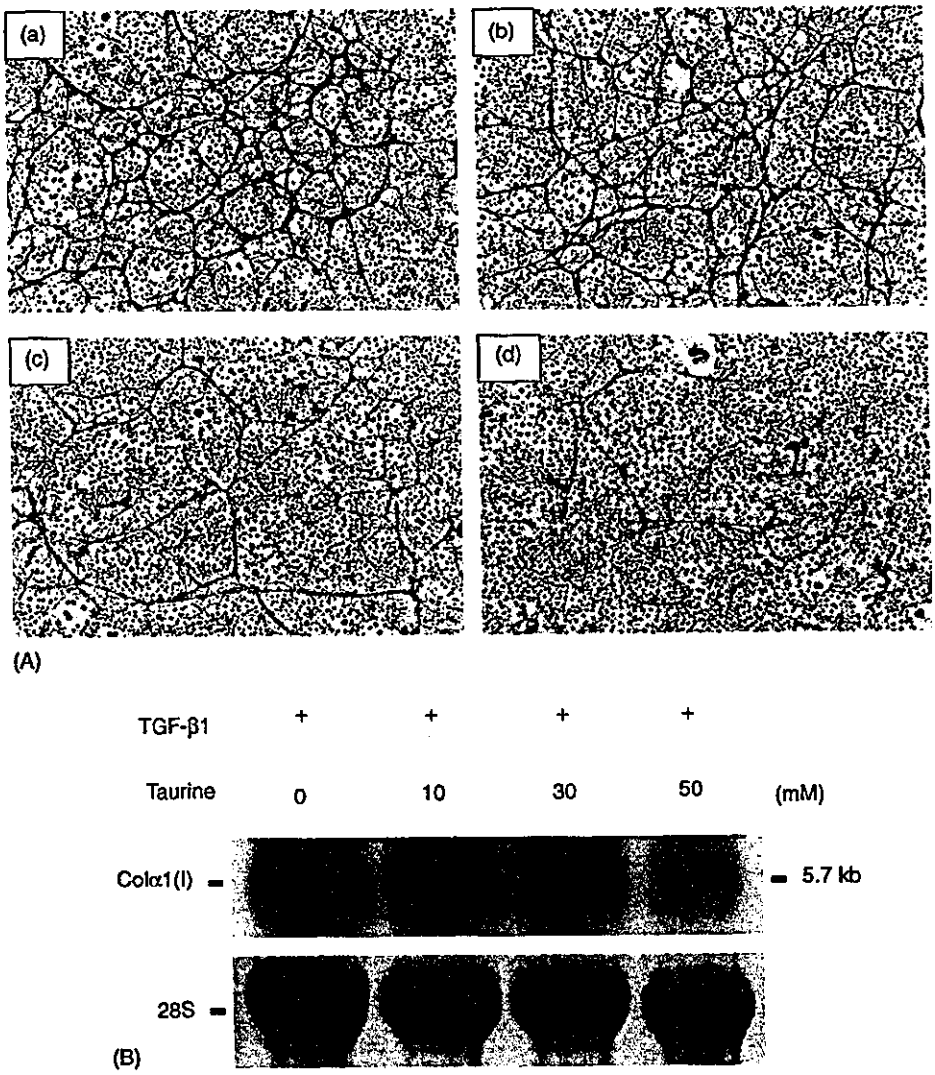

Fig. 4. Taurine inhibited the increase in the formation of the collagen fiber network and procollagen α1(I) expression induced by TGF-β1. (A) M cells were simultaneously treated with TGF-β1 (1 ng/ml) and various concentrations of taurine, and subjected to silver impregnation. (a) M cells treated with 0 mM of taurine, (b) 10 mM, (c) 30 mM, (d) 50 mM. Even in the presence of TGF-β1, exposure to taurine inhibited the formation of the collagen fiber network stimulated by TGF-β1 (magnification 100×). (B) Effect of taurine on the expression of procollagen α1(I) mRNA in the presence of TGF-β1 was evaluated using Northern blot analysis. Treatment with taurine suppressed procollagen α1(I) expression, even when M cells were simultaneously treated with TGF-β1 (1 ng/ml).

with TGF-β1. These results indicate that TGF-β1 may function through autocrine and paracrine mechanisms, and TGF-β-mediated signaling pathways play an important role in the production of collagen fibers in M cells. Although the main source of myofibroblast-like cells and ECM production in the liver is the perisinusoidal stellate cell, TGF-β1 is particularly prominent among the cytokines involved in the activation of HSCs [5] and the stimulation of expression of type I collagen [30,31]. Therefore, M cells could be a useful in vitro system to investigate the mechanisms of TGF-β-mediated fibrogeneis.

The formation of thick a collagen fiber network in M cells was easily identified by stains for collagen, e.g. silver impregnation, and the appearance of collagen fibers surrounding bundles of M cells resembles the histological findings of hepatic fibrosis. Therefore, this culture system is thought to be suitable for screening anti-fibrogenic agents. Recently, several investigations have reported that treatment with a combination of taurine and niacin or taurine alone reduced TGF-β expression and inhibited an increase in type I collagen expression in animal models of bleomycin-induced lung fibrosis or age-related progressive renal fibrosis. respectively [23–25]. In this study, treatment with taurine inhibited the formation of the collagen fiber network and expression of procollagen α1(I) mRNA in a dose dependent manner. This treatment did not affect the production of TGF-β1 by M cells, and, even when M cells were treated with taurine and TGF-β simultaneously, collagen fiber formation and procollagen α1(I) expression were inhibited. Treatment with taurine, however, did not affect either phosphrylation

of Smad2 or expression of Smad7 (data not shown). These results indicate the possibility that taurine suppresses the formation of collagen fiber network and expression of procollagen α1(I) mRNA without affecting TGF-β-mediated signaling pathways. Recently, Akimoto et al. have reported that glycosaminoglycans such as heparin also inhibited collagen fiber network formation in M cells, although collagen synthesis and expression of type I collagen mRNA were not affected by this treatment [32]. Collagen fibers have been shown to interact with some proteoglycans in rat tail tendon, and glycosaminoglycans affected collagen fibril formation from a collagen solution [33,34]. Therefore, heparin may prevent the collagen molecule from interacting with other materials such as proteoglycans, resulting in the inhibition of collagen fiber formation. In the present study, treatment with taurine, however, inhibited not only collagen fiber formation but also procollagen α1(I) expression. Therefore, the anti-fibrogenic effects of taurine are associated with a decrease in collagen production rather than an inhibition of collagen fiber network formation.

In summary, we further characterized M cells, a cell line derived from rat liver cells that actively forms a collagen fiber network. We showed that M cells exhibit both hepatic epithelial- and myofibroblast-like features, and TGF-β1 stimulated collagen production in M cells via autocrine and paracrine mechanisms. Additionally, treatment with taurine inhibited the formation of the collagen fiber network and expression of procollagen α1(I) in M cells. Although further experiments are needed to clarify the characterization of M cells and molecular mechanisms of taurine-induced anti-fibrogenic effects, this culture system is a useful in vitro model of hepatic fibrosis to be used in the investigation of TGF-β-mediated fibrogenesis or screens of various anti-fibrogenic agents.

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Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein

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Abstract

Hepatocyte growth factor (HGF) stimulates liver regeneration and has the potential to be a therapeutic agent for fatal liver diseases, including fulminant hepatic failure and liver cirrhosis. In this study, we investigated the pharmacokinetics of recombinant human HGF, which will be soon available for clinical applications. When recombinant human HGF (0.1 mg/kg) was administered intravenously to normal rats, serum levels of human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the bolus injection, followed by a decrease with a half-life of 2.4 min. Recombinant HGF administered intravenously was distributed primarily to the liver and induced c-Met tyrosine phosphorylation in liver tissues. In comparison, rats given recombinant human HGF via the portal vein exhibited lower serum HGF and an increase in hepatic distribution. Additionally, when compared with normal rats, those with 70% partial hepatectomy or liver cirrhosis showed an increase in serum levels of human HGF with a prolonged half-life. These results suggest that, despite a short half-life, bolus injection of recombinant human HGF may induce therapeutic effect in patients with fatal live disease, and that the dose of this recombinant protein should be modulated according to the degree of liver injury.

Keywords: Hepatocyte growth factor; Pharmacokinetics; Tissue distribution; Serum HGF

1. Introduction

Hepatocyte growth factor (HGF) was originally purified from the plasma of patients with fulminant hepatic failure [1,2], and is one of the major agents that promote hepatocyte proliferation. HGF also functions as a pleiotropic factor, acting as a mitogen, motogen, and morphogen for a variety of epithelial cells (e.g. kidney, intestinal and bronchial cells) as well as endothelial cells by ligation of the c-Met receptor at the cell membrane [3–8]. Additionally, recent investigations have demonstrated that HGF inhibits apoptosis [9–15], and that fibrotic changes in various organs, including the liver and kidneys, were ameliorated by treatment with HGF [16–21]. Thus, HGF plays an important role in the regeneration and repair of injured tissues.

We have previously established an enzyme-linked immunosorbent assay (ELISA) to measure human HGF in serum, and reported that levels of serum human HGF increased in patients with various liver diseases [22]. We have also demonstrated that the measurement of serum HGF is useful for outcome prediction in fulminant hepatic failure [23]. Recombinant human HGF will soon be available for patients with fatal liver diseases, including fulminant hepatic failure, small-for-size grafts in living donor liver transplantation (LDLT), and liver cirrhosis. Since HGF functions as an anti-

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apoptotic or anti-fibrotic factor as well as an agent stimulating hepatocyte proliferation, administration of recombinant HGF is considered to not only induce liver regeneration, but also inhibit disease progression and ameliorate liver cirrhosis. In the present study, we investigated the pharmacokinetics of recombinant human HGF administered intravenously or via the portal vein. We show here that intravenous injection of recombinant human HGF in a bolus induced an increase in the serum levels of human HGF, and that intravenously administered HGF was primarily distributed to the liver. Conversely, compared with intravenous administration, portal vein injection of recombinant human HGF increased hepatic distribution of the protein, whereas serum HGF levels were reduced. We also demonstrate that, despite the short half-life, the single intravenous injection of recombinant HGF induced tyrosine phosphorylation of c-Met in liver tissues.

2. Materials and methods

2.1. Animals

Eight-week-old male Wistar rats were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were maintained under constant room temperature (25 °C) and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the ethical committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after a 1-week acclimation period on a standard diet.

Seventy percent partial hepatectomy was performed according to a modification of the method of Higgins and Anderson [24]. The rats were anesthetized with ether and a two-thirds partial hepatectomy was performed. To induce liver fibrosis, the rats were fed a choline-deficient, L-amino acid-defined (CDAA) diet (Dyets Inc., Bethlehem, PA) for 30 weeks. The development of cirrhosis was confirmed by macroscopic inspection and histological examination [25].

2.2. Measurement of serum human HGF

A silicone-rubber catheter (0.5 mm × 1.0 mm o.d.) was inserted into the jugular vein and saline was administered continuously via the catheter using an infusion pump (0.1 ml/h) to prevent obstruction. Recombinant human HGF (0.1 mg/kg) was injected into inguinal vein or splenic vein in less than 10 s, and sequential blood samples were obtained via the catheter 5, 10, 20, 30, 60, 90 and 120 min after the injection.

2.3. Preparation of tissue extracts

Tissue extracts were prepared as previously described [26] with a slight modification. The various tissues were excised 5 min after the intravenous or intraportal injection of recombinant human HGF (0.1, 0.03 or 0.01 mg/kg), and the wet weight of tissue samples was determined. Fresh tissues were homogenized in cold Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.4% EDTA-2Na and 500 units/ml of aprotinin. The homogenates were centrifuged at 9000 × *g* for 20 min at 4 °C, and then at 105,000 × *g* for 1 h at 4 °C. The supernatants were used for measurement of HGF levels and Western blot analysis.

2.4. Measurement of HGF in sera and tissue extracts

HGF levels in serum and tissue extracts were determined by a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan), in which only human HGF, but not rat HGF, is detected [22,26].

2.5. Western blotting

Tyrosine phosphorylation of c-Met was evaluated by Western blotting. Liver tissues were solubilized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM Na_3Vo_4, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A. Post-nuclear supernatants were precleared with protein A-agarose and immunoprecipitated with anti-c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-agarose. Immunoprecipitated materials were washed five times with 0.1% NP40 and 0.05% sodium deoxycholate and eluted by boiling in Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After blocking membranes with 1% bovine serum albumin, filters were incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibody and subjected to ECL Western blotting detection (Amersham Life Sciences, Buckinghamshire, England).

3. Results

3.1. Changes in serum levels of recombinant human HGF in normal, hepatectomized, and cirrhosis rats

We examined changes in serum levels of human HGF following bolus injection of recombinant human HGF using ELISA (Fig. 1). Our ELISA could not detected endogenous rat HGF. When recombinant human HGF (0.1 mg/kg) was injected into normal rats via inguinal veins in less than 10 s, the level of serum human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the injection (Fig. 1A). Recombinant human HGF disappeared from serum with a half-life of 2.4 min, and the serum HGF decreased to 0.65 ± 0.13 ng/ml after 120 min. These findings indicate that the bolus injection of recombinant HGF induced a considerable increase in serum human HGF, followed by disappearance with a short half-life.

Recombinant human HGF will soon be administered to patients with fatal liver disease, including small-for-size grafts

in LDLT or liver cirrhosis. We therefore investigated the effect of loss of liver volume or development of cirrhosis on the serum levels of recombinant human HGF. When recombinant human HGF (0.1 mg/kg) was injected intravenously into rats with 70% partial hepatectomy, the serum levels of human HGF increased to 341.2 ± 55.1 ng/ml at 5 min (Fig. 1A). A significant increase in serum HGF levels was also observed at 10, 30 and 120 min, and the half-life was prolonged (in comparison to normal rats) to 3.8 min. Similarly, the bolus injection of recombinant human HGF into rats with liver cirrhosis led to significantly elevated levels of serum human HGF at each time point and a prolonged half-life (4.4 min).

Conversely, when recombinant human HGF (0.1 mg/kg) was injected to portal veins in normal rats, the serum level of human HGF was only 17.8 ± 5.94 ng/ml 5 min after the injection (Fig. 1B). This is lower than after the intravenous injection; furthermore, the recombinant human HGF injected via portal vein was rapidly decreased with a half-life of 3.0 min (Fig. 1B). In comparison with normal rats, those with 70% partial hepatectomy exhibited significantly higher

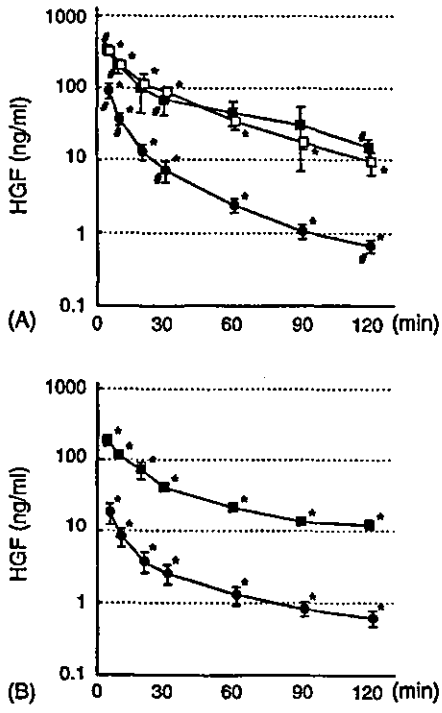

Fig. 1. Serum levels of recombinant human HGF following bolus injection in normal, hepatectomized, and cirrhotic rats. (A) Recombinant human HGF (0.1 mg/kg) was administered intravenously to normal (●: $n=4$), partially hepatectomized (■: : $n=3$–4), and cirrhotic (□: $n=4$) rats, and serum human HGF was determined 5, 10, 20, 30, 60, 90 and 120 min by ELISA as described in Section 2. The serum levels of human HGF at 5 min and its half-life were 89.7 ± 20.6 ng/ml and 2.4 min in normal rats, 341.2 ± 55.1 ng/ml and 3.8 min in rats with partial hepatectomy, and 319.8 ± 22.3 ng/ml and 4.4 min in rats with CDAA diet-induced liver cirrhosis. * and #, $P<0.05$. (B) Recombinant human HGF was injected via the portal vein in normal (●: $n=4$) and partially hepatectomized (■: : $n=3$–4) rats. The serum level of human HGF at 5 min and its half-life were 17.8 ± 5.9 ng/ml and 3.0 min in normal rats and 180.5 ± 29.6 ng/ml and 5.3 min in rats with partial hepatectomy. *, $P<0.05$.

(180.5 ± 29.6 ng/ml at 5 min) levels of serum HGF following portal vein injection of the recombinant protein, and the recombinant protein had a prolonged half-life (5.3 min). These results suggest that the clearance of recombinant human HGF is dependent on liver volume or the number of hepatocytes, and suggest that the recombinant human HGF is trapped primarily in the liver.

3.2. Tissue distribution of recombinant human HGF administered intravenously or via portal vein

To address whether the liver is the primary organ targeted by recombinant human HGF, we examined tissue distribution of recombinant human HGF administered intravenously or via portal vein. Various tissue samples were obtained 5 min after the bolus injection of recombinant human HGF, and the HGF content of the tissues was measured by ELISA. When recombinant human HGF was administered intravenously, the liver, spleen, adrenal gland, and kidneys contained large amounts of human HGF (290.2 ± 38.3, 582.2 ± 205.0, 278.1 ± 114.1 and 101.3 ± 19.3 ng/g wet tissue, respectively) (Fig. 2, open columns). There were small amounts of human HGF in the lungs, pancreas, and large intestine (16.2 ± 3.2, 6.2 ± 0.9, and 2.0 ± 0.2 ng/g wet tissue, respectively). Although the HGF level per tissue weight in spleen was higher than that in liver, we consider human HGF administered intravenously to be primarily distributed to the liver because the weight of the liver is approximately 20-fold larger than that of the spleen. In contrast, when recombinant human HGF was injected via the portal vein, HGF content in the liver tissues increased to 519.5 ± 218.0 ng/g wet tissue (Fig. 2, closed columns). In comparison with intravenous injection, human HGF levels in extra-hepatic organs, including spleen, adrenal gland, kidney, and lungs, were significantly reduced. These results indicate that the liver is the organ predominantly targeted by intravenously administered recombinant human HGF, and the possibility that portal vein injection of the recombinant protein reduces the effects of HGF on extra-hepatic organs.

3.3. Intravenously injected recombinant human HGF effectively induces c-Met tyrosine phosphorylation

Recombinant human HGF, intravenously administered in a bolus, disappeared from serum with a short half-life. Therefore, we investigated whether a single bolus injection of recombinant human HGF induced tyrosine phosphorylation of c-Met, a specific receptor for HGF, in liver tissues (Fig. 3). Various amounts of recombinant human HGF (0.03, 0.1, 0.3 and 1.0 mg/kg) were injected intravenously in less than 10 s. When 0.1 mg/kg or greater recombinant HGF was administered, c-Met tyrosine phosphorylation was induced 30 min after the injection, and expression of c-Met decreased in parallel with the dose of recombinant HGF. Tyrosine phosphorylation of c-Met was close to background in rats given 0.03 mg/kg of recombinant HGF.

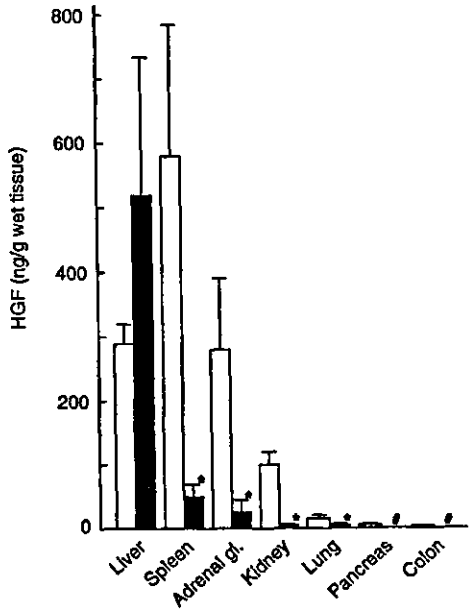

Fig. 2. Tissue distribution of recombinant human HGF administered intravenously or via the portal vein. Various tissue samples were obtained 5 min after bolus injection of recombinant human HGF (0.1 mg/kg). Tissue extracts were prepared and human HGF contents were measured by ELISA as described in Section 2. When recombinant human HGF was injected intravenously, a large amount of human HGF was observed in the liver, spleen, adrenal gland, and kidney (290.2 ± 38.3, 582.2 ± 205.0, 278.1 ± 114.1 and 101.3 ± 19.3 ng/g wet tissue, respectively). The amount was smaller in lung, pancreas, and large intestine (16.2 ± 3.2, 6.2 ± 0.9 and 2.0 ± 0.2 ng/g wet tissue, respectively) (open columns) ($n = 4$). Bolus injection via portal vein significantly reduced the distribution of this protein to extra-hepatic organs (spleen, adrenal gland, kidney, and lung), while HGF content in liver tissues was increased to 519.5 ± 218.0 ng/g wet tissue (closed columns) ($n = 4$). #, HGF content in pancreas and large intestine of rats that received human HGF via the portal vein were not determined. *, $P < 0.05$ in comparison with HGF content in each organ of rats treated intravenously with recombinant human HGF.

3.4. Rats with partial hepatectomy, given recombinant human HGF via the portal vein, exhibited reduced extra-hepatic distribution and increased HGF content in liver tissues

Administration of recombinant human HGF via portal vein is suitable for patients with partial hepatectomy or recipients of LDLT. We therefore investigated the effect of 70% partial hepatectomy on HGF levels in the liver, spleen, and kidneys 5 min after the portal vein injection (Fig. 4). When 0.1 mg/kg HGF was injected via the portal vein in hepatectomized rats, the HGF content in liver, spleen, and kidneys increased to 1262.0 ± 616.6, 82.0 ± 18.3 and 38.5 ± 5.8 ng/g wet tissue, respectively, which is relatively higher than in normal rats (Fig. 2). Administration of a lower dose of recombinant HGF resulted in lower HGF content in liver, spleen, and kidneys. Interestingly, when 0.03 mg/kg of HGF was administered to 70% partial hepatectomized rats via the portal vein, the HGF content in liver tissues (547.8 ± 153.3 ng/g wet tissue) was approximately the same as that in normal rats administered with 0.1 mg/kg of the protein (Figs. 2 and 4). The

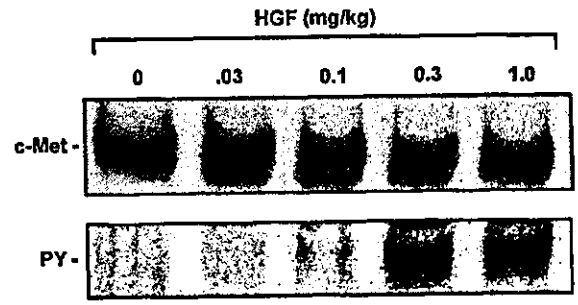

Fig. 3. Bolus injection of recombinant human HGF induced c-Met tyrosine phosphorylation in liver tissue. Recombinant human HGF (0.03, 0.1, 0.3 and 1.0 mg/kg) or PBS was intravenously administered to normal rats. Liver tissues were obtained 30 min after bolus injection. Expression and tyrosine phosphorylation of c-Met were examined by Western blotting, as described in Section 2. Administration of more than 0.1 mg/kg of recombinant human HGF induced c-Met tyrosine phosphorylation in liver tissue, and expression of c-Met decreased in parallel with the dose of recombinant HGF.

dose of recombinant human HGF could be reduced to 30% to treat hepatectomized animals with a remnant liver volume of 30%. These results indicate that when recombinant human HGF is administered to patients with partial hepatectomy or recipients of LDLT, the protein dose should be modulated according to the remnant liver volume.

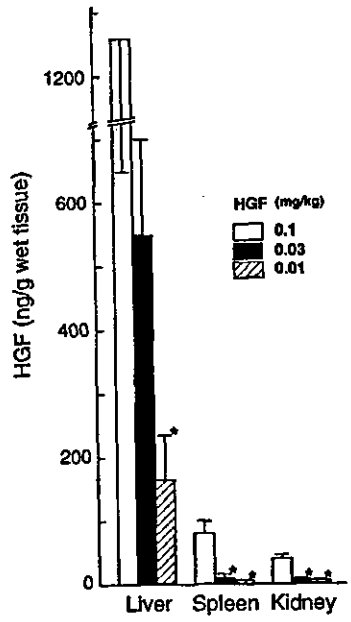

Fig. 4. Tissue distribution of recombinant human HGF administered via the portal vein to rats with 70% partial hepatectomy. Various doses of recombinant human HGF (0.01, 0.03 and 0.1 mg/kg) were administered to rats via the portal vein following 70% partial hepatectomy. The liver, spleen, and kidneys were obtained 5 min after injection, and tissue content of human HGF was measured by ELISA, as described in Section 2. Hepatectomized rats given 0.1 mg/kg of recombinant HGF exhibited an increase in hepatic and extra-hepatic distribution of the protein. When 70% hepatectomized rats were treated with 0.03 mg/kg of recombinant human HGF, its content in liver tissues (547.8 ± 153.3 ng/g wet tissue) was approximately the same as in normal rats given 0.1 mg/kg of recombinant HGF, as shown in Fig. 2. *, $P < 0.05$ in comparison with the HGF content in each organ of hepatectomized rats given recombinant human HGF (0.1 mg/kg).

4. Discussion

HGF is produced from mesenchymal cells as an inactive precursor, pro-HGF [27–29]. After tissue injury, pro-HGF is converted to an active heterodimer, consisting of light and heavy chains, primarily by a specific serine protease, HGF activator (HGFA) [30–32] as well as weakly by blood-coagulation factor XIIa [33]. Recently, HGFA inhibitor (HAI)-1, which was first identified as an inhibitor of HGFA [34], has been reported to act as a reservoir for HGFA in injured tissues [35]. Thus, HGF may play an important role in regeneration and repair of injured tissues, including the liver and intestine. In the present study, we investigated the pharmacokinetics of recombinant human HGF, the heterodimeric active form of HGF. Bolus injection of recombinant human HGF led to its distribution primarily to the liver and effectively induced c-Met tyrosine phosphorylation in liver. Therefore, despite its short half-life, it is possible that intravenously administered recombinant human HGF induces liver regeneration in patients with fatal liver disease. Indeed, intravenous bolus injection of recombinant HGF led to a considerable increase in serum HGF levels (Fig. 1). Treatment with this protein increased liver weight and serum albumin levels in a rat model of dimetylnitrosamine-induced liver injury, in which serum levels of endogenous rat HGF were increased to 2–3 ng/ml, (unpublished data), as well as following partial hepatectomy [36,37]. In addition, a single intravenous injection of recombinant human HGF rescued mice from anti-CD95-induced lethal hepatic failure via its anti-apoptotic effects (unpublished data), and HGF-induced inhibition of apoptosis has been reported in various models of hepatic injury, using HGF gene transfer or HGF protein [9–15]. Thus, when recombinant human HGF is administered to patients with fulminant hepatic failure, the treatment is thought to not only stimulate liver regeneration, but also inhibit disease progression through its anti-apoptotic activity.

When recombinant human HGF was administered intravenously to rats with 70% partial hepatectomy or CDAA diet-induced cirrhosis, serum levels of human HGF were elevated. These results suggest that extra-hepatic distribution of administered HGF is increased in patients with liver disease, leading to adverse side effects. Bolus injection of recombinant HGF actually induced considerable levels of HGF in renal tissue (Fig. 2). However, although HGF has been reported to ameliorate renal fibrosis and dysfunction [16], the repeated injection caused reversible proteinuria (unpublished data). Conversely, bolus injection via the portal vein was capable of delivering recombinant human HGF more specifically to the liver, reducing extra-hepatic distribution of the protein. Nevertheless, even when recombinant human HGF was administered via the portal vein, hepatectomized rats exhibited an increase in HGF levels in the liver, extra-hepatic tissues, and serum. Elevated human HGF contents in the remnant livers may be due to an increase in the amount of administered human HGF per liver volume, and it is possible that, once c-Met receptor and extracellular matrix in liver tissues are saturated, recombinant human HGF leads to an increase in human HGF levels in the serum and extra-hepatic tissues. Therefore, although intraportal injections are thought to reduce the effects on extra-hepatic organs, the dose of recombinant human HGF should be reduced according to the degree of liver injury or remnant liver volume regardless of the route of administration. Indeed, recombinant human HGF administered to rats with 70% partial hepatectomy could be reduced to 30% to achieve the same HGF level in liver tissues as in normal rats (Figs. 2 and 4).

HGF is also known to stimulate liver regeneration and reduce fibrosis in experimental models of liver cirrhosis [17–21], and administration of recombinant human HGF has the potential to become a new modality for cirrhosis patients. However, since lengthy HGF administration is likely required to treat cirrhotic patients, development of an appropriate drug delivery system for liver-specific targeting or long-term release is desirable. Additionally, the potential of HGF to stimulate hepatocellular carcinomas needs to be evaluated before beginning clinical applications.

HGF plays an important role in the repair of injured intestinal mucosa. We have recently reported that administration of recombinant human HGF, using osmotic pumps implanted into peritoneal cavities, resulted in a persistent increase in serum human HGF and facilitated colonic mucosal repair in a rat model of experimental colitis [38]. However, in the present study, the human HGF content of colon tissues was extremely low following intravenous injection of the recombinant protein. Therefore, it is necessary to explore whether repeated injections of human HGF ameliorate intestinal mucosal injury. Alternatively, tissue-specific delivery of the recombinant protein should be established to treat inflammatory bowel disease. Effectiveness of HGF has also been reported in animals with injury of various extra-hepatic organs, including kidney, lung, pancreas, and neurons [16,39–41]. Therefore, development of a delivery system for tissue-specific or long-term release may contribute to expanding the clinical application of recombinant human HGF to diseases of extra-hepatic organs, such as renal sclerosis, pulmonary fibrosis, severe pancreatitis, or neurodegenerative disease.

In conclusion, our findings presented here suggest that although recombinant HGF disappears rapidly from serum, bolus injection of recombinant human HGF may exert a therapeutic effect in patients with fatal liver disease.

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Journal of Gastroenterology

Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response

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Background. In Japan, generally, patients with chronic hepatitis C are aged. The aim of this study was to investigate the effect of interferon (IFN) therapy on the mortality of chronic hepatitis C patients over age 60. ***Methods.*** Seven-hundred and seven patients with histologically proven chronic hepatitis C were enrolled in this study; 649 received IFN therapy (IFN group) and 58 did not (control group). The standardized mortality ratio (SMR) and Cox proportional hazard regression analysis were used to evaluate the effect of IFN on the survival of the patients. ***Results.*** Mean follow-up periods in the IFN and control groups were 5.7 and 6.7 years, respectively. During follow-up, 13 patients in the control group died (7 of liver-related diseases) and 42 in the IFN group died (29 of liver-related diseases). The SMRs of the control and IFN groups were 1.40 (95% confidence interval [CI], 0.76–2.45) and 0.73 (95% CI, 0.52–0.98) for overall death, and 10.70 (95% CI, 4.29–22.05) and 5.05 (95% CI, 3.38–7.26) for liver-related death, respectively. Sustained and transient biochemical responders in the IFN group (SMR, 0.53; 95% CI, 0.01–2.97 and SMR, 3.25; 95% CI, 0.87–8.32, respectively) showed lower liver-related mortality compared with the control group. In patients with sustained virological response, liver-related mortality was also very low (SMR, 0.65; 95% CI, 0.01–3.61). The risk for liver-related death of sustained and transient biochemical responders was also low compared with that of the control group (adjusted risk ratios 0.10 [95% CI, 0.01–0.95] and 0.50 [95% CI, 0.11–2.21], respectively). ***Conclusions.*** These results suggest that IFN treatment could reduce liver-related mortality in chronic hepatitis C patients over age 60, notably in patients showing a biochemical response and in those showing a sustained virological response.

Key words: interferon, chronic hepatitis C, aged, liver-related mortality, standardized mortality ratio

Introduction

A high prevalence of hepatitis C virus (HCV) infection is observed in patients with hepatocellular carcinoma (HCC) in Japan.[1–4] In the early 1990s, interferon (IFN) was introduced, and it is now widely used worldwide, as well as in Japan, for the treatment of patients with chronic hepatitis C. Hitherto, many studies, including our own reports, have shown that IFN therapy reduced the incidence of HCC in patients with chronic hepatitis C.[5–10]

Recently, several groups have studied the effect of IFN therapy on survival in patients with chronic hepatitis C. Most of these studies reported that IFN therapy improved the survival of HCV-related chronic hepatitis and cirrhosis, although some studies did not find any efficacy of IFN therapy on survival.[10–19] We also reported the beneficial effect of IFN therapy on survival in chronic hepatitis C patients. In that report, we also

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showed that the effect of IFN therapy on survival was notable in the patients exhibiting sustained and transient biochemical responses, as well as in those showing sustained virological response.[20]

Many clinical trials showed that IFN therapy resulted in normalization of serum aminotransferase levels and eradication of serum HCV RNA, although a sustained virological response was achieved in a limited number of patients.[21–25] Recently, a combination therapy of ribavirin and IFN, or pegylated IFN, has been shown to have efficacy superior to IFN monotherapy for chronic hepatitis C.[26–28]

Patients in Japan with chronic hepatitis C are, generally, aged.[29,30] Also, patients with HCV-related HCC have been shown to be old, with a peak around age 70.[31] Despite the beneficial effects of IFN therapy or combination therapy of IFN and ribavirin for chronic hepatitis C patients, these treatments have several adverse effects which are not tolerable, especially for aged patients who have illnesses other than liver disease.[32] If IFN therapy does not prolong life expectancy in aged patients with chronic hepatitis C, the indications for IFN therapy in these patients may be very limited. Therefore, it is very important to investigate whether IFN therapy could improve survival in aged patients with chronic hepatitis C.

The aim of this study was to evaluate the effect of IFN therapy on mortality in aged patients with chronic hepatitis C. We conducted a multicenter, large-scale, retrospective cohort study of chronic hepatitis C patients over 60 years of age.

Patients and methods

Patients

We found previously that IFN therapy improved the survival in patients with chronic hepatitis C.[20] Of the 2954 patients with chronic hepatitis C in that study, we enrolled 707 patients over age 60 in the present study, to investigate the effect of IFN therapy on mortality in aged patients. Accordingly, the inclusion criteria were the same as those of the previous study: (1) histological diagnosis of chronic hepatitis or cirrhosis; (2) no history of clinical signs, at entry into the study, of complications of cirrhosis, i.e., ascites, jaundice, encephalopathy, or variceal bleeding; (3) no evidence of HCC at entry into the study, as assessed by ultrasonography and/or computed tomography; (4) absence of serum hepatitis B surface antigen; (5) absence of coexisting liver diseases, such as autoimmune hepatitis or primary biliary cirrhosis; (6) absence of excessive alcohol consumption (>80 g/day); and (7) absence of human immunodeficiency virus antibodies.[20]

The IFN group comprised 649 patients who had started IFN therapy between 1992 and 1997 and had received a 4- to 12-month course of IFN, which was initiated within 1 month after liver biopsy. None of the patients had received IFN therapy before entry into this study. The control group consisted of 58 patients who had received liver biopsies between 1986 and 1997, but who did not undergo IFN therapy.

Biochemical responses to IFN therapy were categorized as follows. Patients whose alanine aminotransferase (ALT) levels decreased to the normal range during therapy and remained normal for up to 24 weeks after the end of the therapy were considered to have a sustained biochemical response. Patients whose ALT levels decreased to the normal range by the end of therapy, remained normal during therapy, but returned to abnormal levels during the 24 weeks following the end of the IFN therapy were considered to have a transient biochemical response. All other ALT patterns were classified as showing biochemical non-response. A sustained virological response was defined as persistent HCV RNA negativity during IFN therapy and follow-up. Patients showing positive HCV RNA after IFN therapy were classified as virological non-responders.

Follow-up

Abdominal ultrasonography or computed tomography and biochemical examinations, including α-fetoprotein, were carried out before a liver biopsy and every 3 to 6 months during follow-up, equally in the IFN and control groups. The starting date of follow-up for patients in the control and IFN groups was defined as the date of liver biopsy. Follow-up data that were not available were collected from the resident registry of the local municipal office. In the patients residing in Osaka whose follow-up data were not obtained, the Osaka Cancer Registry was used, and the data were available until the end of 1999.[6] Therefore, it was decided to use the date of death or the end of 1999 as the end of follow-up. Because the longest observation period of the patients in the IFN group was 96 months, only the follow-up data for the first 96 months were considered in the control group. Causes of death were divided into liver-related and liver-unrelated deaths. Causes of liver-related death included HCC, liver failure, and esophageal variceal bleeding.

Informed consent was obtained from each patient included in the study. The study protocol was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and was approved by the Ethics Committee of the Osaka University Graduate School of Medicine.

Table 1. Baseline characteristics of the interferon and control groups

	Interferon group						
	Virological response		Biochemical response				
	Sustained response (n = 161)	Non-response (n = 484)	Sustained response (n = 206)	Transient response (n = 144)	Non-response (n = 299)	Total (n = 649)	Control group (n = 58)	P value
Age (years; mean ± SD)	63.6 ± 3.0	63.3 ± 2.9	63.8 ± 3.1	63.0 ± 2.8	63.1 ± 2.8	63.3 ± 2.9	64.1 ± 3.1	0.06
Age distribution (years; %)								
60–64	67.7	71.1	63.6	75.0	72.9	70.4	56.9	0.03
≥65	32.3	28.9	36.4	25.0	27.1	29.6	43.1	
Male/Female	110/51	272/212	134/72	80/64	171/128	385/264	31/27	0.38
Histologic staging score (%)								
0	0.6	0.2	0.5	0.0	0.3	0.3	5.2	0.06
1	24.8	18.2	27.7	25.0	12.4	20.0	31.0	
2	29.2	27.7	26.7	28.5	28.8	28.0	20.7	
3	39.8	46.9	40.3	39.6	50.5	44.8	31.0	
4	5.6	7.0	4.9	6.9	8.0	6.8	12.1	
ALT (IU/l; mean ± SD)	113 ± 82	107 ± 68	110 ± 86	87 ± 45	117 ± 69	108 ± 71	105 ± 80	0.75

Histological evaluation

In all patients, liver biopsy was undertaken before IFN therapy. Sections were stained with hematoxylin-eosin and Azan-Mallory and analyzed by two pathologists in a blinded manner. For the assessment of liver histology, the classification of Desmet et al.[33] was used.

Statistical analysis

To compare the distribution of age at liver biopsy and histological staging between the IFN and control groups, the Wilcoxon rank-sum test was used. Differences in age at liver biopsy and ALT between the two groups was assessed for significance by Student's t-test. The χ^2 test was used to compare sex differences. The Kaplan-Meier method was used to compare the cumulative survival rates in the IFN and control groups.

We compared the observed number of deaths with the expected number of deaths, which was calculated by applying sex-, 5-year age, 5-year calendar time, and cause-specific mortality rates for the general population in Japan, as prepared by the Statistics and Information Department, Japan Ministry of Health and Welfare.[34] The standardized mortality ratio (SMR) was expressed by dividing the observed number of deaths by the expected number of deaths. Survival was also analyzed by Cox proportional hazards regression. For analysis, age, sex, stage of liver fibrosis (stages 0,1/2/3/4), time of liver biopsy (until 1992/after 1993), and IFN therapy were used as variables. SMRs and hazard risk ratios were expressed with 95% confidence intervals (CIs).

Data analysis was performed with the SAS/PC statistical package (SAS Institute, Cary, NC, USA). All reported P values were two-sided, and a P value of less than 0.05 was considered to be significant.

Results

Baseline characteristics

In the IFN group, 206 patients (31.7%) had a sustained biochemical response, 144 (22.2%) had a transient biochemical response, and 299 patients (46.1%) were biochemical non-responders. Four sustained biochemical responders whose serum HCV RNA was not examined during follow-up were excluded from the analysis. Accordingly, 161 patients (25.0%) of the 645 IFN-treated patients were classified as sustained virological responders. Table 1 shows the baseline characteristics of the IFN and control groups. Age at entry, sex, histologic staging score, and serum ALT level did not differ between the two groups. The proportion of patients more than 65 years of age in the control group was higher than that in the IFN group (P = 0.03).

Table 2. Cumulative survival rate calculated from overall deaths

	Interferon group					
	Virological response		Biochemical response			
	Sustained response	Non-response	Sustained response	Transient response	Non-response	Total	Control group
Mean follow-up period (years; mean ± SD)	5.7 ± 1.6	5.7 ± 1.7	5.6 ± 1.7	5.7 ± 1.8	5.8 ± 1.6	5.7 ± 1.7	6.7 ± 1.7
4-Year survival rate	99.3%	96.2%	98.4%	99.2%	95.0%	97.0%	93.0%
8-Year survival rate	94.6%	86.8%	94.3%	93.0%	83.4%	88.7%	73.9%
P Value[a]	<0.001	0.0197	<0.001	0.0036	0.1212	0.0031	

[a] The log rank test was used to determine the difference against the control group

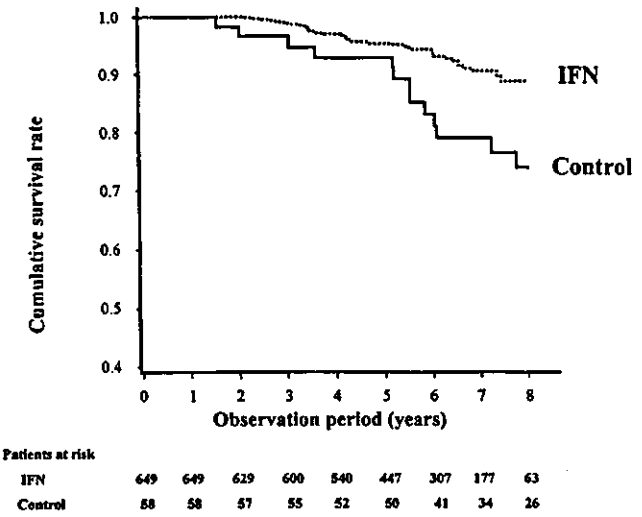

Fig. 1. Cumulative survival rates in the interferon (*IFN*; *dotted line*) and control (*solid line*) groups. Log-rank test of the two curves showed a significant difference between the two groups ($P = 0.003$)

Cumulative survival and cause of death

The mean follow-up periods of the IFN and control groups were 5.7 and 6.7 years, respectively. The mean follow-up periods of the patients with each response in the IFN group are shown in Table 2. Figure 1 shows the cumulative survival rates of the IFN and control groups, estimated by the Kaplan-Meier method. The 8-year survival rates of the IFN and control groups were 88.7% and 73.9%, respectively (log-rank test; $P = 0.003$; Table 2). The cumulative survival rates of sustained virological responders were significantly higher than those for virological non-responders (log-rank test; $P = 0.02$). The 8-year survival rates of sustained virological responders and virological non-responders were 94.6% and 86.8%, respectively (Table 2). The cumulative survival rates of both the sustained and transient biochemical responders were significantly higher than that of the biochemical non-responders (log-rank test; $P = 0.007$ and $P = 0.049$; Fig. 2). The 8-year survival rates of sustained and transient biochemical respon-ders and biochemical non-responders were calculated to be 94.3%, 93.0% and 83.4%, respectively (Table 2).

During follow-up, 42 of the 649 IFN-treated patients and 13 of the 58 control patients died. The numbers of liver-related and liver-unrelated deaths in the IFN and control groups are shown in Table 3. Liver-related deaths corresponded to 69% of all deaths (29/42) in the IFN group and 54% of all deaths (7/13) in the control group. HCC was the major cause of liver-related deaths in both groups. Only one liver-related death (17%) was found in the deaths of sustained biochemical respond-

Table 3. Causes of death in the interferon and control groups

	Interferon group					Control group (n = 58)
	Virological response		Biochemical response			
	Sustained response (n = 161)	Non-response (n = 484)	Sustained response (n = 206)	Transient response (n = 144)	Non-response (n = 299)	Total (n = 649)
All deaths (n)	4	38	6	6	30	42	13
Liver-related deaths (n)	1	28	1	4	24	29	7
Hepatocellular carcinoma	1	25	1	3	22	26	5
Other causes	0	3	0	1	2	3	2
Liver-unrelated deaths (n)	3	10	5	2	6	13	6

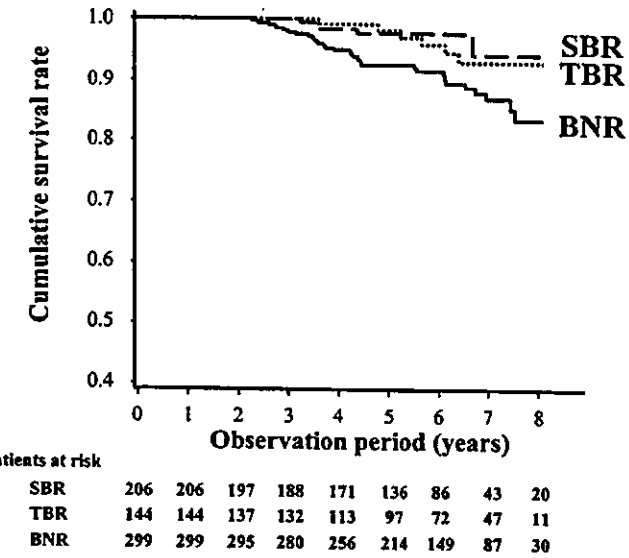

Fig. 2. Cumulative survival rates in the IFN-treated patients, categorized by sustained biochemical response (*SBR*; *dashed line*), transient biochemical response (*TBR*; *dotted line*), and biochemical non-response (*BNR*; *solid line*). Log-rank test showed significant differences between SBR and BNR (P = 0.007) and between TBR and BNR (P = 0.049)

ers. In the control group, 6 patients died of causes other than liver disease; 2 patients died of stomach cancer; 1 patient each died of lung cancer, colon cancer, and cerebral infarction; and in 1 patient, the cause of death was a traffic accident. In the IFN group, we identified 13 liver-unrelated deaths; 4 patients died of stomach cancer; 3 died of lung cancer; and 1 each died of breast cancer, colon cancer, esophageal cancer, pneumonia, chronic renal failure, and multiple myeloma.

Cox proportional hazard regression analysis

Cox proportional hazard regression analysis revealed that the risk of overall death in the IFN group was lower than that in the control group, with a marginally significant difference (risk ratio, 0.37; 95% CI, 0.13–1.05; Table 4). The patients with a sustained virological response had a low risk of overall death (risk ratio, 0.15; 95% CI, 0.04–0.59) compared with the control group. Sustained and transient biochemical responders also showed low risks of overall death (risk ratio, 0.18; 95% CI, 0.05–0.65; and risk ratio, 0.24; 95% CI, 0.07–0.87). The risk of liver-related death in the IFN group was similar to that in the control group (Table 4). However, the patients with sustained virological and biochemical response had a low risk of liver-related death compared to the control group (risk ratio, 0.12; 95% CI 0.01–1.16 and risk ratio, 0.10; 95% CI, 0.01–0.95, respectively). In transient biochemical responders, the risk ratio for liver-related deaths was 0.50 (95% CI, 0.11–2.21).

Table 4. Risk ratios for death in interferon and control groups

	All deaths			Liver-related deaths		
	Risk ratio	95% CI	*P* value	Risk ratio	95% CI	*P* value
Control group	1.00			1.00		
IFN group	0.37	0.13–1.05	0.06	0.80	0.25–2.53	0.71
Sustained virological response	0.15	0.04–0.59	0.01	0.12	0.01–1.16	0.07
Virological non-response	0.44	0.16–1.23	0.12	0.97	0.31–3.05	0.96
Sustained biochemical response	0.18	0.05–0.65	0.01	0.10	0.01–0.95	0.05
Transient biochemical response	0.24	0.07–0.87	0.03	0.50	0.11–2.21	0.36
Biochemical non-response	0.54	0.19–1.53	0.24	1.26	0.40–4.03	0.69

Age, sex, time of liver biopsy (until 1992/after 1993) and histologic staging score were adjusted in the Cox proportional hazard analysis

SMR

The SMRs in the IFN and control groups are shown in Table 5 and Fig. 3. In the control group, overall mortality was slightly higher than that in the sex- and age-matched general population (SMR, 1.40; 95% CI, 0.76–2.45). On the other hand, overall mortality in the IFN group was significantly lower compared with that of the general population (SMR, 0.73; 95% CI, 0.52–0.98). Liver-related mortality was high in the control group (SMR, 10.70; 95% CI, 4.29–22.05), and it was also high in the IFN group (SMR, 5.05; 95% CI, 3.38–7.26), although it was half of that in the control group. In the patients with sustained virological response, liver-related mortality (SMR, 0.65; 95% CI, 0.01–3.61) was very low compared with that in the control group, and it was similar to that for the general population. On the contrary, liver-related mortality was high in virological non-responders (SMR, 6.71; 95% CI, 4.46–9.70).

In terms of biochemical response, the SMRs for liver-related death of sustained and transient biochemical responders in the IFN groups were low compared with that in the control group (SMR, 0.53; 95% CI, 0.01–2.97 and SMR, 3.25; 95% CI, 0.87–8.32, respectively). In the patients with biochemical non-response, liver-related mortality was high, and was equal to that in the control group (SMR, 9.12; 95% CI, 5.84–13.57).

The IFN group showed lower liver-unrelated mortality than the general population (SMR, 0.25; 95% CI, 0.13–0.43), whereas the control group had liver-unrelated mortality similar to the general population (SMR, 0.71; 95% CI, 0.26–1.55).

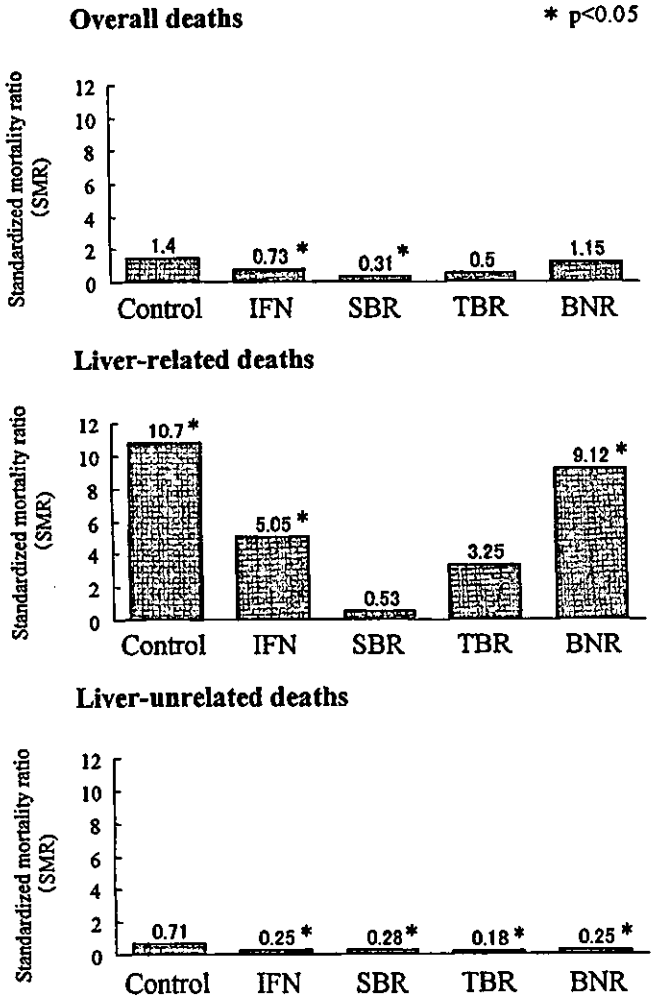

Fig. 3. Standardized mortality ratios (*SMRs*) for overall, liver-related, and liver-unrelated deaths. *SBR*, sustained biochemical response; *TBR*, transient biochemical response; *BNR*, biochemical non-response. When the SMR did not include unity, we considered the difference from the expected number of deaths to be significant

Discussion

There have been a few reports regarding the effect of IFN therapy on survival in chronic hepatitis C patients.[10,16–19] Yoshida et al.[17] reported that IFN therapy had a preventive effect on liver-related death, bringing