Table 5. Standardized mortality ratios (SMRs) in interferon and control groups

	All deaths			Liver-related deaths			Liver-unrelated deaths		
	Observed	Expected	SMR (95% CI)	Observed	Expected	SMR (95% CI)	Observed	Expected	SMR (95% CI)
Control group	13	9.1	1.40 (0.76–2.45)	7	0.7	10.70 (4.29–22.05)	6	8.4	0.71 (0.26–1.55)
Interferon group	42	57.8	0.73 (0.52–0.98)	29	5.7	5.05 (3.38–7.26)	13	52.0	0.25 (0.13–0.43)
Sustained virological response	4	15.8	0.25 (0.07–0.65)	1	1.5	0.65 (0.01–3.61)	3	14.3	0.21 (0.04–0.61)
Virological non-response	38	41.7	0.91 (0.64–1.25)	28	4.2	6.71 (4.46–9.70)	10	37.6	0.27 (0.13–0.49)
Sustained biochemical response	6	19.5	0.31 (0.11–0.67)	1	1.9	0.53 (0.01–2.97)	5	17.6	0.28 (0.09–0.66)
Transient biochemical response	6	12.1	0.50 (0.18–1.08)	4	1.2	3.25 (0.87–8.32)	2	10.9	0.18 (0.02–0.66)
Biochemical non-response	30	26.2	1.15 (0.77–1.64)	24	2.6	9.12 (5.84–13.57)	6	23.5	0.25 (0.09–0.55)

A difference from the expected number of deaths was considered significant when the 95% confidence interval (CI) of SMR did not include unity

about improved survival of chronic hepatitis C patients, as assessed by multivariate analysis and SMR. Recently, we also reported that IFN therapy improved survival by preventing liver-related deaths in patients with chronic hepatitis C, in a multicenter, large-scale, retrospective cohort study.[20] In that study, we showed that liver-related mortality, as well as overall mortality, was much higher in untreated patients than in IFN-treated patients, as assessed by SMR. Furthermore, we found that patients showing sustained and transient biochemical responses to IFN therapy had a very low risk of death compared with untreated patients.

In this study, we evaluated the effect of IFN therapy on survival in patients over 60 years of age with histologically proven chronic hepatitis C, by SMR and by risk ratio calculated by Cox proportional hazard regression analysis. Compared with the general population, liver-related mortality was high in the IFN-treated patients (SMR, 5.05), but it was much lower than that in the control group (SMR, 10.70). Yoshida et al.[17] also examined the effect of IFN therapy on liver-related mortality in chronic hepatitis C patients over 60 years of age in their large-scale retrospective cohort study, and reported that the SMR for liver-related death in IFN-treated patients was much lower than that in the untreated patients, which was consistent with our result. In our IFN group, sustained virological responders and sustained biochemical responders had very low liver-related mortality (SMR, 0.65 and 0.53, respectively), which was equal to that in the sex- and age-matched general population. Multivariate regression analysis also showed that IFN therapy reduced the risk of liver-related death in sustained virological responders by 88% and in sustained biochemical responders by 90%. The overall mortality in the control group was not high (SMR, 1.40), whereas that in the IFN group was significantly lower in comparison with the sex- and age-matched general population (SMR, 0.73). These results may reflect a selection bias due to the nature of the liver biopsy procedure, which was undergone by all of the patients in our study. This kind of selection bias may occur, as aged patients sometimes have illnesses other than liver disease, which make a liver biopsy difficult. Furthermore, IFN-treated patients had a significantly lower risk of liver-unrelated mortality compared with the untreated patients. It seems likely that this may be attributed not to the beneficial effect of IFN therapy on liver-unrelated mortality but to a selection bias in using IFN; only the patients who had no serious diseases, such as cardiovascular disease, received IFN therapy. However, our study indicated that IFN therapy could reduce liver-related mortality, particularly in patients with sustained virological or biochemical response.

In the patients with a transient biochemical response, liver-related mortality was low when compared with the

control group, as assessed by SMR. The SMR of the transient biochemical responders (3.25; 95% CI, 0.87–8.32), which included unity, was lower than that in the control patients (10.70; 95% CI, 4.29–22.05). Similarly, the risk ratio for liver-related death in transient biochemical responders was 0.50, although this was not significant. On the other hand, SMR, as well as the risk of liver-related death estimated by multivariate analysis in the biochemical non-responders (SMR, 9.12; adjusted risk ratio, 1.26), was similar to that in the control patients. These data suggest that a reduction in liver-related mortality by IFN therapy can be expected in patients showing a transient biochemical response. Retreatment or long-term treatment with IFN might lead to an improved survival rate in transient biochemical responders, although such treatment may not be easy with some aged patients.

There was no difference between the baseline characteristics of the IFN and control groups, except for the age distribution. However, because our study was a retrospective cohort study, it had some limitations. Because the time at liver biopsy in the control group was earlier than that in the IFN group, lead-time bias may have existed. The survival of the IFN group could be higher than that of the control group. To minimize this bias, 5-year time-specific mortality rates for the general population were prepared in the SMR analysis. Furthermore, the time at liver biopsy was included as a variable for the multivariate analysis. Another limitation of our study is the small number of patients in the control group compared with the IFN group. This limitation may also be overcome by calculating the SMRs of the IFN and control groups, representing the ratio of the observed number of deaths to the expected number of deaths, calculated after taking sex-, calendar time-, and cause-specific mortality rates for the general population into consideration. The beneficial effect of IFN therapy on survival in the aged patients with chronic hepatitis C resulting from the SMR analysis was consistent with that of the Cox proportional hazard regression analysis.

In conclusion, we showed in this study that IFN therapy reduced liver-related mortality in aged patients with chronic hepatitis C, especially in those exhibiting a biochemical response and in those showing a sustained virological response. IFN therapy is recommended for aged patients with chronic hepatitis C in whom a biochemical response or a sustained virological response can be expected, after screening for diseases other than chronic hepatitis C.

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Editorial

Sustained activation of epidermal growth factor receptor in cholangiocarcinoma: a potent therapeutic target?

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See Article, pages 808–814

Cholangiocarcinoma is a malignant neoplasm originating from epithelium of the biliary tree and is associated with a high rate of mortality [1]. It is the second most common primary malignant tumor of the liver after hepatocellular carcinoma, and comprises approximately 10% of all hepatobiliary malignancies. Specific mechanisms underlying the cellular and molecular pathogenesis of cholangiocarcinoma remain unclear. However, evidence suggesting that alterations in critical growth factor pathways may contribute to the development of this highly lethal cancer is accumulating.

Extrahepatic cholangiocarcinoma, which arises from large bile ducts, is more frequent than intrahepatic cholangiocarcinoma arising from small bile ducts. Both the large and small bile ducts, however, are lined with cholangiocytes [2]. Recently, large cholangiocytes arising from large bile ducts, but not small cholangiocytes from smaller bile ducts, have been shown to transport bile acids [3], and the heterogeneity of cholangiocytes along the biliary tract is now a well-established entity [4]. Therefore, in contrast to small bile duct cholangiocytes, specific characteristics of large bile duct cholangiocytes likely predispose to the development of this cancer.

One known risk factor for ductal cholangiocarcinoma is chronic inflammation, including primary sclerosing cholangitis (PSC), *Clonorchis sinensis* and *opisthorchis viverrini* infections (liver flukes), Caroli's disease, congenital choledochal cysts, and chronic intrahepatic lithiasis [5]. Also, in Japan, hepatitis C infection is frequently found in patients with intrahepatic cholangiocarcinoma [6]. Chronic inflammation is associated with cytokine generation from both inflammatory cells and cholangiocytes. A key proinflammatory cytokine is interleukin 6 (IL-6). A marked increase in serum levels of IL-6 has been observed in patients with cholangiocarcinoma. Moreover, IL-6 stimulates growth of cholangiocarcinoma cells by a mitogen-activated protein kinase (MAPK) signaling pathway, suggesting that IL-6 is a potent mitogen for cholangiocytes and cholangiocarcinoma cells [7,8].

Ductal cholangiocarcinomas often grow within and along the bile duct lumen. These tumor cells can, therefore, survive and proliferate even in the toxic environment of bile. Alpini et al. have demonstrated that bile acids stimulate the proliferation of cholangiocytes and cholangiocarcinoma cells through a phosphatidylinositol 3-kinase (PI-3K)-dependent pathway [9–11]. PI-3K is stimulated by a number of mitogenic receptor tyrosine kinases. Recently, several investigations have reported that bile acids functionally transactivate the epidermal growth factor receptor (EGFR) in primary cultured hepatocytes or cholangiocarcinoma cells [12,13]. Also, bile acids increase cellular protein levels of myeloid cell leukemia protein-1, a potent anti-apoptotic protein of the Bcl-2 family, via EGFR activation [14]. Therefore, transactivation of EGFR is thought to be an important mechanism by which bile acids stimulate cholangiocyte proliferation. The mechanism underlying bile acid-mediated EGFR transactivation is potentially both ligand-dependent and -independent [12,14]. EGFR is usually activated by several ligands including EGF, transforming growth factor (TGF)-α, and heparin-binding EGF-like growth factor. Werneburg et al. have recently reported that bile acids activate EGF receptor via a TGF-α-dependent mechanism in a human cholangiocyte cell line [15]. Additionally, since matrix metalloproteinase (MMP) activity is required for TGF-α release from cellular membranes and ligand function, bile acid-induced EGFR activation and cell growth are inhibited by an MMP inhibitor. Conversely, ligand-independent signaling of

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EGFR occurs by processes, usually overexpression, which promote receptor oligomerization in the plasma membrane [16]. Indeed, overexpression and aberrant function of EGFR have been observed in several human carcinomas, and hence, the EGFR-tyrosine kinase is a selective therapeutic target for inhibiting tumor growth. Although EGFR signaling plays an important role in the pathogenesis and progression of cholangiocarcinoma, the characteristics of EGFR signaling in cholangiocarcinoma cells have not been fully understood.

Yoon and co-workers report details of the mechanism for overexpression of EGFR and its sustained activation in human cholangiocarcinoma cells in this issue of the Journal [17]. They found that, when cholangiocarcinoma cells were treated with EGF, EGFR activation was sustained resulting in extended MAPK activation and growth stimulation. Also, they demonstrated that, even though EGFR is promptly ubiquitinated following ligand-induced activation, its internalization is either defective or delayed in cholangiocarcinoma cells, leading to prolonged expression of EGFR on the plasma membrane after exposure to EGF, thereby sustaining its activation. A ubiquitin ligase, c-Cbl, is activated by EGFR, and c-Cbl-mediated ubiquitination of active EGFR is essential for receptor degradation [18]. Also, formation of the endocytotic complex at the plasma membrane, comprised of c-Cbl, CIN85 (Cbl-interacting protein of 85 K) and endophilins, is critical for receptor internalization [19,20]. Inhibition of these interactions has recently been shown to block EGFR internalization, delay receptor degradation, and enhance EGFR signaling transduction, without perturbing c-Cbl-direct receptor ubiquitination [21]. Thus, the findings in this issue indicate the possibility that human cholangiocarcinoma cells lack interaction between c-Cbl and CIN85/endophilin.

Cyclooxygenase (COX)-2, a crucial rate-limiting enzyme in prostaglandin metabolism, has been shown to inhibit apoptosis, promote angiogenesis, and facilitate cellular growth in a variety of malignancies [22]. This enzyme can be induced by various proinflammatory cytokines in biliary epithelial cells, and has been implicated in cholangiocarcinogenesis [23]. Recently, bile acid transactivation of EGFR has been reported to induce COX-2 expression through a MAPK cascade [13]. In this issue, Yoon et al. also demonstrate that EGFR kinase inhibitors decreased COX-2 expression in human cholangiocarcinoma cells, and that cell growth was significantly inhibited by treatment with EGFR kinase inhibitors as well as a COX-2 inhibitor [17]. Therefore, either EGFR or COX-2 are promising drug targets for cholangiocarcinoma, and their inhibition has the potential to treat and prevent this cancer. However, expression of c-Met, a specific receptor for hepatocyte growth factor (HGF), is also observed in a significant percentage of human cholangiocarcinomas by immunohistochemistry [24], and the growth of human cholangiocarcinoma cells have been shown to be stimulated by HGF as well as IL-6 [25]. Additionally, treatment with HGF or IL-6 induced the rapid phosphorylation of cytosolic phopholipase A2 (cPLA2), another crucial rate-limiting enzyme in prostaglandin metabolism, and HGF- and IL-6-induced proliferation of cholangiocarcinoma cells was significantly inhibited by either a cPLA2 or COX-2 inhibitor [25]. Therefore, although clinical studies of anti-EGFR therapeutic strategies, including the EGFR tyrosine kinase inhibitor ZD1839 (Iressa; AstraZeneca Pharmaceuticals LP, Wilmington, DE), have shown clinical responses in a variety of solid tumors [16], it is necessary to carefully evaluate whether therapeutic targeting of only EGFR can induce sufficient effect on established cholangiocarcinoma. Conversely, a selective COX-2 inhibitor, celecoxib, being developed as a potential treatment for rheumatoid arthritis and osteoarthritis, can be used as a potent chemopreventive agent in different types of cancer [26]. The role of selective COX-2 inhibitors as chemopreventive agents for cholangiocarcinoma needs to be assessed in patients with PSC, which is one risk factor for cholangiocarcinoma [1]. Also, the mechanisms and the molecular pathology of the chemopreventive effects should be further clarified. However, extensive investigations to understand alterations in tyrosine kinase receptor signaling including EGFR and the role of COX-2 expression in cholangiocarcinoma cells or cholangiocarcinogenesis would provide a potent therapeutic or chemopreventive modality for cholangiocarcinoma, a nefarious neoplasm of the bile duct apparatus.

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Rapid Communication

Orally administered Kampo medicine, Juzen-taiho-to, ameliorates anemia during interferon plus ribavirin therapy in patients with chronic hepatitis C

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Background. Interferon plus ribavirin (IFN/Rib) therapy is currently standard treatment for chronic hepatitis C. Hemolytic anemia, however, is a serious side effect of this treatment, requiring reductions in or complete withdrawal of ribavirin. ***Methods.*** We retrospectively investigated the effect of the Kampo medicine Juzen-taiho-to (TJ-48), which contains bone marrow-stimulating compounds, on anemia in 67 patients with chronic hepatitis C, who received IFN/Rib therapy. ***Results.*** The reduction in hemoglobin levels was significantly ameliorated in TJ-48-treated patients ($P < 0.05$). Consequently, only 13% (4/32) of TJ-48-treated patients received altered doses of ribavirin, while the ribavirin dose had to be reduced or withdrawn in 43% (15/35) of patients in the absence of TJ-48 administration ($P < 0.001$). ***Conclusions.*** These results indicate the possibility that oral administration of TJ-48 supports IFN/Rib therapy without necessitating ribavirin reduction or withdrawal.

Key words: chronic hepatitis C, interferon, ribavirin, Juzen-taiho-to

Introduction

Interferon plus ribavirin (IFN/Rib) combination therapy has come to be the frontline therapy for patients in Japan with chronic hepatitis C who exhibit genotype 1b and a high viral load.[1] Approximately 35% of patients, however, exhibit 25% or greater decreases in hemoglobin levels, which often occurs within the first 4 weeks of this treatment.[2] The anemia observed during IFN/Rib therapy is due to hemolysis;[3] when hemoglobin levels are markedly reduced, reduction or withdrawal of ribavirin is required. The Kampo medicine Juzen-taiho-to (TJ-48) traditionally ameliorates anemias; recent investigations have reported that TJ-48 contained substances that stimulated hematopoietic stem cell growth,[4] T-cell-mediated suppression of melanocytic tumor growth,[5] and the inhibition of tumor metastases.[6] Clinically, TJ-48 is used in Japan to alleviate anemia associated with autologous blood preservation and anticancer chemotherapy. We therefore evaluated the effect of TJ-48 administration on anemia induced by IFN/Rib therapy.

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Subjects and methods

We examined 67 patients with chronic hepatitis C receiving IFN/Rib therapy, after we obtained the appropriate informed consent. All patients were less than 70 years old. The selection criteria for participation were a positive test for anti-hepatitis C virus (HCV) antibodies, serum HCV RNA levels greater than 200 kilo international units (KIU)/ml by quantitative polymerase chain reaction (PCR) assay (Amplicor GT-HCV Monitor Version 2.0; Roche Molecular Systems, Pleasanton, CA, USA) (defined as high viral load), persistently high serum alanine transaminase (ALT) concentrations, and hemoglobin concentrations of 13 mg/dl. Of the 67 patients who agreed to receive TJ-48 administration, 32 received IFN/Rib therapy in combination with TJ-48, while the remaining 35 patients were given IFN/

Table 1. Baseline characteristics of chronic hepatitis C patients receiving IFN/Rib therapy with or without additional TJ-48 administration

	Non-TJ-48-treated patients	TJ-48-treated patients	*P* value[b]
Number of patients	35	32	
Sex (M/F)	23/12	19/13	0.962
Age (years)	54.8 ± 9.0[a]	54.7 ± 7.8[a]	0.622
Body weight (kg)	64.3 ± 10.0[a]	62.8 ± 10.7[a]	0.569
Viral load (KIU/ml)	620 ± 269[a]	780 ± 782[a]	0.258
HCV genotype			
1a	0	0	
1b	28	27	1.000
2a	5	4	
2b	2	2	
Dose of ribavirin			
600 mg/day	15	16	0.628
800 mg/day	20	16	

[a] Data values are expressed as means ± SD
[b] *P* values were analyzed by Fisher's exact test or Student's *t*-test

Rib therapy alone. Interferon alpha-2b (Intron-A; Schering-Plough International, Kenilworth, NJ, USA) was administered at a dose of 6 million units (MU) daily for the initial 2 weeks. Following the 2-week induction therapy, the interferon alpha-2b (600 MU) administration was reduced to three times a week for 22 weeks. Ribavirin (Rebetol; Schering-Plough International) was administered for 24 weeks, at a dose of 800 mg/day for patients weighing 60 kg or more; a ribavirin dose of 600 mg/day was used for patients weighing less than 60 kg. TJ-48 (7.5 g/day) administration began at either the initiation of IFN/Rib therapy or 1 to 4 weeks before treatment. We evaluated sequential changes in hemoglobin and in serum ALT levels. We also monitored the number of patients exhibiting marked decreases in hemoglobin (less than 10 g/dl) that necessitated the reduction or withdrawal of ribavirin within the first 12 weeks of IFN/Rib therapy. Statistical parameters were ascertained using Statview software (SAS Institute, Cary, NC, USA). Statistical analyses were performed by Student's *t*-test or Fisher's exact test. *P* Values of less than 0.05 were considered to be statistically significant.

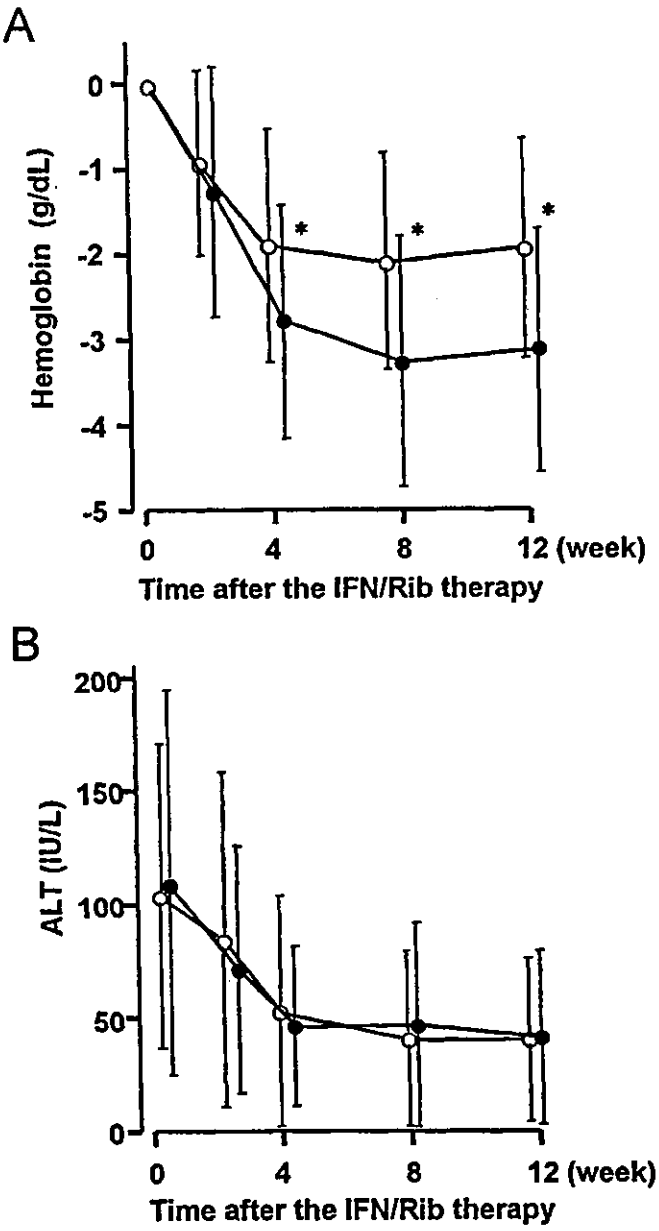

Fig. 1A,B. Effects of Juzen-taiho-to (TJ-48) on **A** hemoglobin and **B** alanine transaminase (*ALT*) levels in patients with chronic hepatitis C treated with interferon plus ribavirin (*IFN/Rib*) therapy. The levels of hemoglobin (**A**) and serum ALT (**B**) were examined before (week 0) and at 2, 4, 8, and 12 weeks after IFN/Rib therapy began in TJ-48-treated (*open circles*) and non-TJ-48-treated (*closed circles*) patients. The reduction in hemoglobin levels during IFN/Rib therapy were significantly ameliorated by TJ-48 administration, while decreases in serum ALT were not affected. * $P < 0.05$ in comparison with hemoglobin levels in TJ-48-treated patients at each time point

Results and discussion

There were no significant differences in the backgrounds (sex, age, body weight, viral load, HCV genotype, initial dose of ribavirin) of the TJ-48-treated and -untreated patients, although this was not a randomized control study (Table 1). The reduction in hemoglobin levels in the TJ-48-treated patients was significantly less than that observed in the non-TJ-48-treated patients 4, 8, and 12 weeks into the therapy course ($P < 0.05$; Fig. 1A). There were no significant differences in either the

Table 2. Number of patients who required reduction or withdrawal of ribavirin

Initial Hb level (g/dl)	Non-TJ-48-treatled patients		TJ-48-treated patients	
	Total	Requirement for ribavirin reduction or withdrawal	Total	Requirement for ribavirin reduction or withdrawal
≧17	1	○	1	○
16.0–16.9	1	●	1	○
15.0–15.9	9	●●●●●○○○○	7	○○○○○○○
14.0–14.9	12	●●●●○○○○○○○○	9	●●○○○○○○○
13.0–13.9	12	●●●●●○○○○○○○	⌊14	●●○○○○○○○○○○○○
Total no. of patients	35	●: 15 (43%) vs ○: 20 (57%)*	32	●: 4 (13%) vs ○: 28 (87%)*

* $P < 0.001$ (Fisher's exact test)
Closed and *open circles*, patients who required and did not require, respectively, reduction or withdrawal of ribavirin

sequential changes in serum ALT levels (Fig. 1B) or in viral load (data not shown). When patients were given IFN/Rib therapy in the absence of TJ-48, 15 out of 35 patients (43%) exhibited severe anemia, with hemoglobin levels of less than 10 g/dl; these patients, consequently, required reduction or withdrawal of ribavirin (Table 2). Even in patients whose hemoglobin levels prior to IFN/Rib therapy were greater than 15 g/dl, IFN/Rib therapy induced severe anemia in 55% (6/11) of the non-TJ-48-treated patients, leading to drug reduction or withdrawal. In contrast, only 13% (4/32) of the TJ-48-treated patients required reduction or withdrawal of ribavirin due to severe anemia ($P < 0.001$). In patients with initial hemoglobin levels greater than 15 g/dl, IFN/Rib therapy was continued without either reduction or withdrawal of ribavirin when administered in combination with TJ-48.

Kampo medicines have been used, primarily in China, to treat a wide variety of diseases for over 2000 years. Recently, basic and clinical research has begun to conclusively demonstrate the effectiveness of Kampo medicines, justifying their wide use in clinical practice in Japan. TJ-48 is a spray-dried preparation of a fixed proportion of ten crude drugs listed in the Japanese Pharmacopoeia. The major components are: paeoniflorin (derived from peony root), β-eudesmol (from *atractyllodes lancea* rhizome), ginsenoside (Rb1; from ginseng root), and glycyrrhizic acid (from glycyrrhiza root). Although the effects of TJ-48 are thought to be based on the combined effects of these components, the fatty acids included in TJ-48 play an important role in bone marrow stimulation.[4] The quality of TJ-48 is determined by measuring the content of paeoniflorin and glycyrrhizic acid by high-performance liquid chromatography. In Japan, TJ-48 is widely used for the treatment of anemia and the prevention of chemotherapy- and radiotherapy-induced bone marrow suppression, as well as for the suppression of tumor metastases and recurrence. Therefore, it is likely that treatment with TJ-48 stimulates bone marrow cells, resulting in the alleviation of anemia induced by IFN/Rib therapy. Alternatively, TJ-48 has been shown to affect T-cell-mediated immunity.[5] The efficacy of IFN/Rib therapy for chronic hepatitis C is associated with a vigorous peripheral blood T-helper (Th)1 cell response,[7] indicating the possibility that administration of TJ-48 prevents anemia via immunological mechanisms.

The findings presented here indicate that, although the anemia observed during IFN/Rib therapy, which is likely to be hemolytic, is reversible, the concurrent administration of TJ-48 ameliorates the decreases in hemoglobin levels in patients treated with interferon and ribavirin, contributing to the success of this combination therapy.

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Inhibition of hepatocyte growth factor induction in human dermal fibroblasts by interleukin-1 and its prevention by interferon-γ

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Abstract

Hepatocyte growth factor (HGF) is one of the vital factors for liver regeneration. HGF production is induced by the activation of protein kinase A and protein kinase C-mediated pathways, interleukin (IL)-1, tumor necrosis factor (TNF)-α, and epidermal growth factor (EGF) in mesenchymal cells. We here report that IL-1 and TNF-α, hitherto regarded as HGF inducers, potently inhibited HGF production stimulated by other HGF inducers. IL-1α, IL-1β, and TNF-α alone had minimal stimulating effects on HGF production in human dermal fibroblasts, but they strongly inhibited production of HGF induced by cholera toxin, 8-bromo-cAMP, EGF, and phorbol 12-myristate 13-acetate (PMA). Moreover, although the high level of HGF production in MRC-5 cells was enhanced by PMA and less markedly by IL-1β, HGF production in MRC-5 cells treated with PMA plus IL-1β was less than that in the cells treated with PMA alone. In the presence of interferon (IFN)-γ, however, cholera toxin- and 8-bromo-cAMP-induced HGF production was not inhibited by IL-1β. Pretreatment of cells with IL-1β suppressed the phosphorylation of cAMP-responsive element-binding protein induced by cholera toxin but not that induced by 8-bromo-cAMP. Taken together, our results indicate that IL-1 inhibited HGF production stimulated by various inducers, including protein kinase A-activating agents, and that IFN-γ overcame this inhibition of induction of HGF production.

Keywords: Hepatocyte growth factor; Interleukin-1; Tumor necrosis factor-α; cAMP; Induction; Dermal fibroblast; MRC-5

Hepatocyte growth factor (HGF), also known as scatter factor, was initially isolated as a mitogenic factor for adult rat hepatocytes in primary culture [1–5]. There is mounting evidence that HGF is a vital factor for liver regeneration [6]. Levels of HGF in the plasma and liver of rats that had been exposed to carbon tetrachloride or that had been partially hepatectomized increase markedly prior to liver regeneration [7,8]. These increases are accompanied by elevations of the mRNA levels of this factor in the liver, spleen, and lung [9–11]. Continuous administration of neutralizing anti-HGF antibody to rats treated with carbon tetrachloride inhibits liver regeneration [12]. Elevated HGF in plasma and liver after hepatic injury may play a role in proliferation of hepatocytes.

HGF is mainly produced by mesenchymal cells such as fibroblasts and smooth muscle cells [13,14]. HGF production in those cells in vitro is induced by a variety of cytokines, hormones, and chemicals, although an actual inducer of HGF in the injured liver has not yet been identified. Those HGF inducers include activators of protein kinase A (PKA) and protein kinase C, interleukin (IL)-1,

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tumor necrosis factor (TNF)-α, interferon (IFN)-γ, estrogen, ascorbic acid, okadaic acid, norepinephrine, a scatter factor-inducing factor, and various growth factors [15–19]. In contrast, HGF production is inhibited by transforming growth factor (TGF)-β, glucocorticoids, 1,25-dihydroxyvitamin D_3, and retinoic acid [20–24]. Among those downregulators, TGF-β has the most potent inhibitory effect on HGF production.

Many lines of evidence support the concept that TGF-β is a physiological inhibitor of liver regeneration, which ceases after enlargement of the liver remnant to its original mass. TGF-β inhibits DNA synthesis in cultured adult rat hepatocytes and in regenerating rat liver [25,26]. DNA synthesis in regenerating rat liver peaks at 24 h after partial hepatectomy and then gradually declines and is negligible at 72 h, when the maximal level of TGF-β is reached [27]. Thus, TGF-β may act as a dual inhibitor of hepatocyte proliferation in regenerating liver: it not only directly inhibits DNA synthesis of hepatocytes but also suppresses production of HGF. Recently, IL-1 was also proposed to be a potential downregulator of liver regeneration, based on its inhibitory effect on hepatocyte proliferation both in vitro and in vivo, and on results of studies examining IL-1 expression after partial hepatectomy and in Kupffer cell-depleted rats subjected to partial hepatectomy [28–30]. HGF-inducing activity of IL-1, however, is puzzling, because this activity may counteract a potential suppressor effect of IL-1 on liver regeneration.

In view of reports regarding cross-talk between different signaling pathways, we have examined the combined effects of IL-1 and various other HGF inducers on HGF production employing human dermal fibroblasts as model cells. Here we report that IL-1α and IL-1β strongly inhibited HGF production stimulated by other HGF inducers rather than enhancing it. Moreover, we found that the inhibitory effect of IL-1 was completely overcome by IFN-γ but not by either IFN-α or IFN-β.

Materials and methods

Reagents. Dulbecco's modification of Eagle's minimum essential medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Recombinant human IL-1α and IL-1β were purchased from Otsuka Pharmaceutical (Tokyo, Japan) and R&D Systems (Minneapolis, MN), respectively. Natural human TNF-α, IFN-α, and IFN-γ were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Cholera toxin and 8-bromo-cAMP were obtained obtained from Sigma Chemical (St. Louis, MO). Recombinant human IFN-β was purchased from PBL Biomedical Laboratories (Piscataway, NJ). Rabbit anti-phosphorylated cAMP-responsive element-binding protein (phospho-CREB) and anti-CREB (CREB-1) antibodies were obtained from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Other reagents were obtained from previously reported sources [31].

Cell culture. Normal human dermal fibroblasts isolated from 200 individual neonatal donors were obtained from Cell Systems and used between seventh and tenth passages. MRC-5 human embryonic lung fibroblasts were obtained from American Type Culture Collection. The cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air as described previously [20].

Determination of HGF levels in conditioned media. Human dermal fibroblasts and MRC-5 lung fibroblasts, trypsinized and suspended in the medium described in the previous section, were seeded in 96-well plates (Nunc) at a density of 1.8×10^4 cells/cm^2 (0.17 ml/well). After reaching confluence, the medium was replaced with the same fresh medium or that containing IL-1α, IL-1β or TNF-α, and the cultures were incubated for 1 h. An HGF inducer, 10 pM cholera toxin, 1 mM 8-bromo-cAMP, 30 nM phorbol 12-myristate 13-acetate (PMA) or 10 ng/ml of epidermal growth factor (EGF), was then added. The conditioned medium was collected after incubation of the cells for a further 24 h (PMA-treated human dermal fibroblasts), 72 h (cholera toxin-, 8-bromo-cAMP- or EGF-treated human dermal fibroblasts) or 48 h (MRC-5 cells), unless stated otherwise, and was frozen at −30 °C for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [32], with a slight modification [17], except that biotinylated goat anti-human HGF antibody (R&D Systems) and horseradish peroxidase–streptavidin conjugate (Zymed Laboratories) were used as a detection antibody and a horseradish peroxidase conjugate, respectively.

Northern blot analysis. The medium of confluent human dermal fibroblasts grown in 9-cm dishes (Nunc) was replaced with the same fresh medium or that containing IL-1α, IL-1β or TNF-α, and the cultures were incubated for 1 h. Cholera toxin was then added. After incubating for a further 40 h, total RNA was isolated from the cells using RNA-Bee (TEL test). Northern blotting was performed as described previously [31].

Western blot analysis. The medium of confluent human dermal fibroblasts grown in 24-well plates (Nunc) was replaced with the same fresh medium, and the cultures were incubated for 15 h. The cells were then treated with IL-1β for 12 h, and cholera toxin or 8-bromo-cAMP was added. After incubating for 1.5 h with cholera toxin or for 15 min with 8-bromo-cAMP, cells were washed three times with phosphate-buffered saline and lysed by adding 30 μl SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). Lysates were boiled for 10 min, briefly sonicated, and centrifuged. Protein in extracts was determined as described previously [33]. Western blotting was performed as described previously [33]. Briefly, protein aliquots (each 10 μg) were separated by 10% SDS–PAGE and transferred electrophoretically to Immobilon-P transfer membranes (Millipore). The membranes were then probed with rabbit anti-phospho-CREB or anti-CREB antibody and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) and detected with ECL Plus Western blotting detection reagents (Amersham Biosciences).

Statistical analysis. All results are expressed as means and SEM of several independent experiments. Data in two groups were analyzed by unpaired Student's t test. Multiple comparison of the data was done by Dunnett's t test. P values less than 5% were regarded as significant.

Results

Inhibition by IL-1α, IL-1β, and TNF-α of HGF production stimulated by various inducers in human dermal fibroblasts

IL-1 effectively induces HGF production in MRC-5 and IMR-90 human embryonic lung fibroblasts but it

is a weak inducer of HGF production in human dermal fibroblasts [17,34]. HGF production in human dermal fibroblasts is markedly induced by cholera toxin, 8-bromo-cAMP, EGF, and PMA [20,31,35]. The effects of IL-1α and IL-1β on HGF production stimulated by these inducers were tested. Both IL-1α and IL-1β dose-dependently inhibited cholera toxin-induced HGF production (Fig. 1A). Ninety percent inhibition was observed with concentrations of IL-1α and IL-1β higher

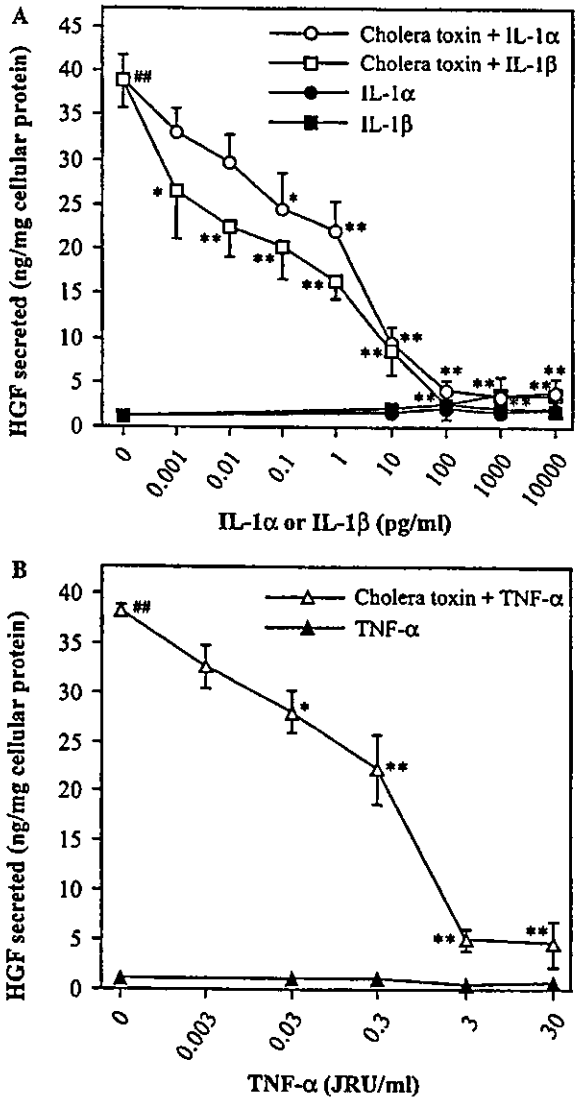

Fig. 1. Inhibition of cholera toxin-induced HGF production in human dermal fibroblasts by IL-1α, IL-1β, and TNF-α. Confluent human dermal fibroblasts were pre-incubated for 1 h with the indicated concentrations of IL-1α (A), IL-1β (A) or TNF-α (B). The cells were then incubated for 72 h with or without 10 pM cholera toxin in the presence or absence of IL-1α, IL-1β or TNF-α. The data are means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's t test), as compared with the values of the respective controls (medium alone and cholera toxin alone). $^{\#\#}P < 0.01$ (Student's t test), as compared with the values of the medium alone.

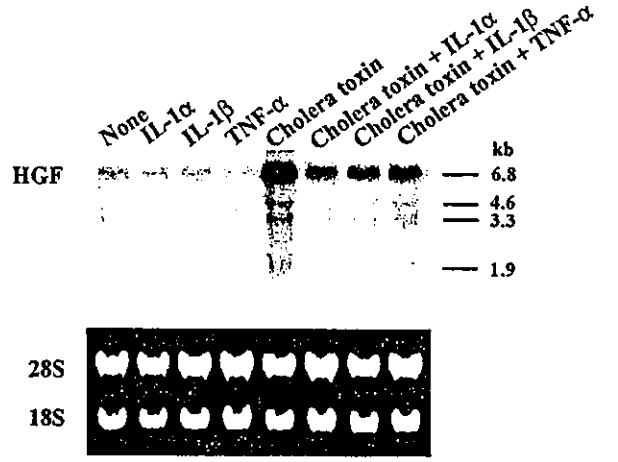

Fig. 2. Inhibition by IL-1α, IL-1β, and TNF-α of upregulation of HGF gene expression by cholera toxin in human dermal fibroblasts. Confluent human dermal fibroblasts were pre-incubated for 1 h with or without IL-1α (0.1 ng/ml), IL-1β (0.1 ng/ml) or TNF-α (3 JRU/ml). The cells were then incubated for 40 h with or without 10 pM cholera toxin in the presence or absence of IL-1α, IL-1β or TNF-α. Autoradiographs and fluorescence photographs are representative of three independent experiments.

than 0.1 ng/ml. When IL-1β was added to the cells 10 or 24 h after cholera toxin instead of being added 1 h before cholera toxin, potent inhibition of the induction of HGF production was still observed (data not shown). TNF-α also caused strong inhibition, demonstrating 50% inhibition at about 0.3 JRU/ml (Fig. 1B). Effects of IL-1α, IL-1β or TNF-α alone were minimal (Figs. 1A and B). 8-Bromo-cAMP-induced HGF production was inhibited by the three cytokines as potently as was cholera toxin-induced HGF production (data not shown). EGF- and PMA-induced HGF production was also inhibited by IL-1α, IL-1β, and TNF-α, but their inhibitions of EGF-induced HGF production were less potent than those of cholera toxin-induced HGF production and their inhibitions of PMA-induced HGF production were always partial (data not shown). The inhibitory effects of IL-1α, IL-1β, and TNF-α on HGF production induced by cholera toxin, 8-bromo-cAMP, and EGF were comparable to those of TGF-β1 and dexamethasone (data not shown).

The effects of IL-1α, IL-1β, and TNF-α on constitutive and induced HGF gene expression in human dermal fibroblasts determined 40 h after the addition are shown in Fig. 2. IL-1α, IL-1β, and TNF-α did not significantly affect the constitutive HGF mRNA level, but they significantly decreased HGF gene expression upregulated by cholera toxin.

Inhibition of PMA-induced HGF production by IL-1β in MRC-5 cells

MRC-5 cells constitutively produce large amounts of HGF, the production of which is induced by IL-1 or

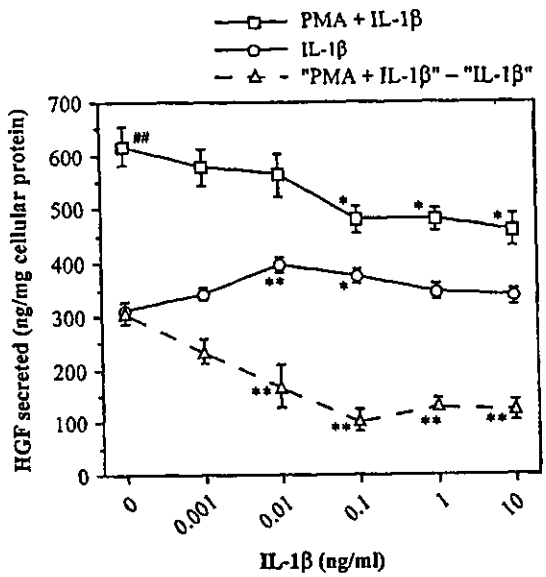

Fig. 3. Inhibition of PMA-induced HGF production in MRC-5 cells by IL-1β. Confluent MRC-5 cells were pre-incubated for 1 h with or without the indicated concentrations of IL-1β. The cells were then incubated for 48 h with or without 10 nM PMA in the presence or absence of IL-1β. The data are means ± SEM of three independent experiments. A dotted line shows differences between HGF production in cells treated with PMA plus IL-1β and HGF production in cells treated with IL-1β alone. $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's t test), as compared with the values of the respective control (medium alone, inducer alone or inducer alone minus medium alone). $^{\#\#}P < 0.01$ (Student's t test), as compared with the values of the medium alone.

PMA [13,34]. Induction of HGF production by the latter was greater than that by the former (Fig. 3). The amount of HGF produced in cells treated with PMA plus IL-1β was significantly less than that in cells treated with PMA alone, indicating that IL-1β inhibited PMA-induced HGF production in MRC-5 cells (Fig. 3). When differences between HGF production in cells treated with PMA plus IL-1β and HGF production in cells treated with IL-1β alone were calculated, IL-1β caused a substantial decline in the increment of HGF production by PMA (Fig. 3).

Cholera toxin-induced but not 8-bromo-cAMP-induced phosphorylation of CREB is inhibited by IL-1β

The mechanisms underlying the inhibition of cholera toxin- or 8-bromo-cAMP-induced HGF production by IL-1β were next examined. The transcription factor CREB is important for the activation of transcription of many cAMP-responsive genes. Phosphorylation of Ser 133 is required for CREB-mediated transcription [36]. Thus, we determined the effect of IL-1β on the phosphorylation of CREB in human dermal fibroblasts treated with or not treated with cholera toxin. Twelve-hour pretreatment with IL-1β markedly inhibited the phosphorylation of CREB in cells incubated for 1.5 h with cholera toxin but not the phosphorylation of CREB in cells incubated for 15 min with 8-bromo-cAMP (data not shown).

Attenuation by IFN-γ of IL-1β-caused inhibition of HGF induction

We reported previously that IFN-γ, a proinflammatory cytokine like IL-1 and TNF-α, augments 8-bromo-cAMP- or cholera toxin-induced HGF production in human dermal fibroblasts [37]. Thus, the effect of

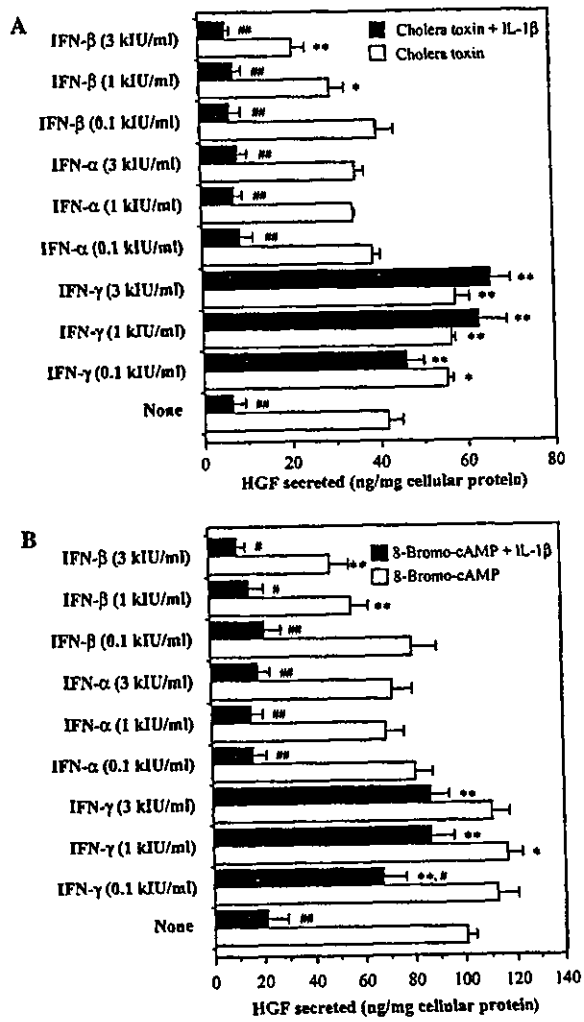

Fig. 4. The suppressive effect of IL-1β on cholera toxin- or 8-bromo-cAMP-induced HGF production in human dermal fibroblasts is overcome by IFN-γ but not by either IFN-α or IFN-β. Confluent human dermal fibroblasts were pre-incubated for 2 h with or without IFN-γ, IFN-α or IFN-β and further incubated for 1 h with or without 0.1 ng/ml IL-1β (A) and 0.03 ng/ml IL-1β (B) in the presence or absence of IFNs. The cells were then incubated for 72 h with or without 10 pM cholera toxin (A) or 1 mM 8-bromo-cAMP (B) in the presence or absence of IFNs and IL-1β. The data are means of three independent experiments. Bars indicate SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's t test), as compared with the values of the inducer alone or the inducer plus IL-1β. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (Student's t test), as compared with the values of the inducer alone or the inducer plus the same concentration of the respective IFNs.

IFN-γ on IL-1β-caused downregulation of cholera toxin- or 8-bromo-cAMP-induced HGF production was examined. In the presence of IFN-γ, IL-1β did not show any inhibitory effect on cholera toxin-induced HGF production, although it did so in the presence of the same doses of IFN-α or IFN-β (Fig. 4A). IL-1β did not cause downregulation of 8-bromo-cAMP-induced HGF production in cells treated with IFN-γ either (Fig. 4B). Furthermore, IL-1β did not downregulate cholera toxin-induced HGF gene expression in the presence of IFN-γ (data not shown).

Discussion

IL-1 has been regarded as an inducer of HGF production in various cells [17,34], but the data shown in this report clearly demonstrated that IL-1α and IL-1β potently inhibited HGF production stimulated by various inducers in human dermal fibroblasts and PMA-induced HGF production in MRC-5 cells, although the potency of their inhibition varied depending on the HGF inducer. HGF production induced by PKA-activating agents such as cholera toxin and 8-bromo-cAMP was most sensitive to IL-1-caused inhibition, and hence IL-1 inhibited cholera toxin- and 8-bromo-cAMP-induced HGF production as strongly as did TGF-β 1 or dexamethasone. Upregulation of HGF mRNA expression by cholera toxin was also suppressed by IL-1α and IL-1β. HGF mRNA expression in regenerating rat liver after partial hepatectomy peaks at 12 h after the operation and then gradually declines, being one-fourth of the peak at 48 h [10]. Expression of IL-1α and IL-1β mRNAs was upregulated at 24 and 48 h after the operation [29]. Since IL-1 showed strong inhibition of HGF induction even when added 24 h after cholera toxin, IL-1 may be effective even if it acts on HGF-producing cells after stimulation with an HGF inducer. Therefore, it seems likely that IL-1α and IL-1β contribute to the downregulation of HGF mRNA expression at 24 and 48 h after the operation. Downregulation of HGF production may play a role in retardation of hepatocyte proliferation in regenerating liver.

IL-1 has recently been proposed to be a potential downregulator of hepatocyte proliferation, terminating the surge of DNA synthesis induced after partial hepatectomy. It is a negative regulator of hepatocyte proliferation both *in vitro* and *in vivo* [28,29]. A conditioned medium of non-parenchymal cells isolated from regenerating rat liver between 24 and 48 h after partial hepatectomy contained an additional non-TGF-β inhibitor of hepatocyte proliferation identified as IL-1 [29]. Administration of IL-1β to rats diminished the replicative surge of hepatocyte DNA synthesis after partial hepatectomy [29]. In contrast, administration of an IL-1 receptor antagonist prior to partial hepatectomy resulted in a marked increase in hepatocyte proliferation in these animals [38]. Moreover, liver regeneration was augmented after partial hepatectomy in rats following selective depletion of Kupffer cells, which are the major IL-1-producing cells in the liver [30]. Thus, IL-1β may play a role as a dual inhibitor of liver regeneration: it not only directly inhibits hepatocyte proliferation but also suppresses induction of the potent hepatocyte mitogen HGF.

Only a few other instances of inhibitory effects of IL-1 on a cAMP action are known: IL-1 inhibited 8-bromo-cAMP-induced decidualization in human endometrial stromal cells and dibutyryl cAMP-induced CRE-driven luciferase expression in human airway smooth muscle cells [39,40]. The molecular mechanisms responsible for the inhibitory effects of IL-1 are largely unknown. The mechanism underlying IL-1β-caused inhibition of cAMP-dependent HGF induction was not elucidated in this study either, but IL-1β may inhibit the ability of protein kinase A-activating agents to induce expression of the HGF gene through events downstream of CREB phosphorylation because IL-1β did not retard 8-bromo-cAMP-induced phosphorylation of CREB. Our results are consistent with recent findings of Lahiri et al. [40] that were obtained using human airway smooth muscle cells. They showed that IL-1β attenuated isoproterenol-induced CREB phosphorylation, but not dibutyryl cAMP-induced CREB phosphorylation, although IL-1β inhibited both isoproterenol-induced and dibutyryl cAMP-induced CRE-driven luciferase expression. Since IL-1 inhibited HGF production induced by EGF and PMA as well as cAMP-induced HGF production, it is possible that one target of IL-1's action is on a signaling pathway for HGF induction common to those inducers.

In the presence of IFN-γ, IL-1β did not inhibit cAMP-dependent HGF induction, suggesting that IFN-γ antagonizes the inhibitory effect of IL-1β. It is possible that they interact in the liver because IL-1β is capable of inducing IFN-γ production in rat hepatocytes in primary culture [41]. There have been some reports of inhibitory effects of IFN-γ on IL-1 actions, although their positive interactions have also been shown. Barrios-Rodiles and Chadee [42] reported antagonistic effects of IFN-γ on IL-1β-induced cyclooxygenase-2 expression and prostaglandin E_2 production in human macrophages. A recent study by Takayanagi et al. [43] showed that IFN-γ induces rapid degradation of tumor necrosis factor receptor-associated factor 6 (TRAF6), an adaptor protein for receptors of several cytokines such as IL-1 and RANKL, in mouse bone marrow cells, resulting in inhibition of the RANKL-induced activation of nuclear factor-κB and c-Jun NH_2-terminal kinase. Inhibition by IL-1β of cholera toxin-induced phosphorylation of CREB, however, was not attenuated in cells treated with IFN-γ. This sug-

gests that IL-1β signaling in human dermal fibroblasts is not blocked by IFN-γ treatment. Thus, it seems likely that IFN-γ overcame IL-1β-caused inhibition of HGF induction not through a direct antagonistic effect but through a more complexed mechanism.

In conclusion, the results of this study demonstrated that IL-1 inhibited HGF production stimulated by various inducers, including PKA-activating agents, and that IFN-γ overcame this inhibition of induction of HGF production, and the results suggest that IL-1 inhibits HGF production induced by PKA-activating agents through events downstream of CREB phosphorylation.

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