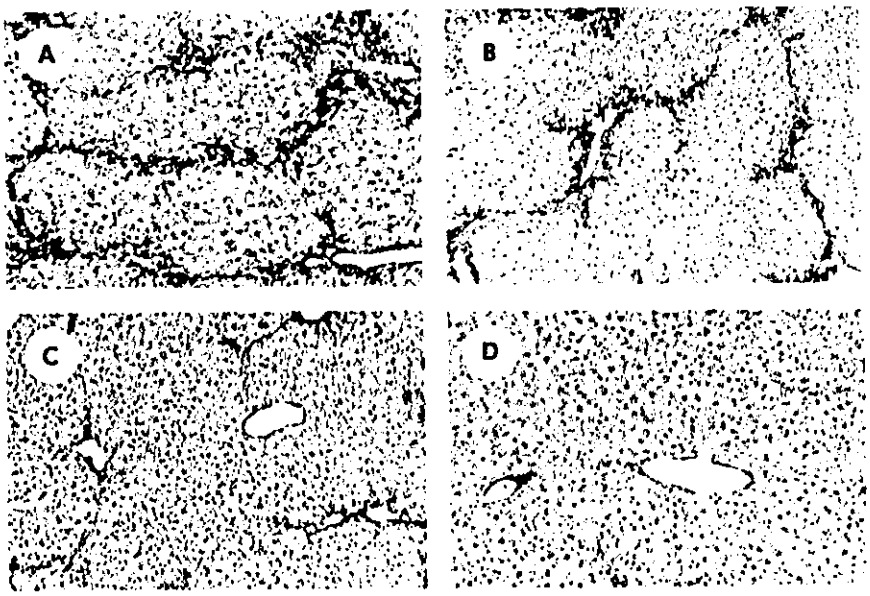


**Figure 5** Immunohistochemical analysis of α smooth muscle actin (α-SMA). Immunopositive cells of α-SMA were significantly reduced in the livers of R-1mAb (B), R-2mAb (C), and the combination of R-1mAb and R-2mAb treated groups (D) compared with the control group (A) (G1) (magnification ×40).

effects of R-1mAb and R-2mAb on the acute liver injury and early liver fibrogenesis step. It has been shown that Masson's trichrome positive connective tissue accumulation could be observed on day 7 after $CCl_4$ treatment.[47] Two or seven days after $CCl_4$ treatment, R-1mAb and R-2mAb did not alter ALT levels in the liver (data not shown). This indicated that the inhibitory effect of mAbs was not a secondary response to a cytoprotective effect against $CCl_4$. Body and liver weights when the mice were killed were not significantly different between the control, R-1mAb treated, and R-2mAb treated groups (data not shown).

### Neovascularisation

To examine whether the inhibitory effects of R-1mAb and R-2mAb were associated with suppression of neovascularisation in the liver, we evaluated the angiogenic response during liver fibrosis development. We performed a preliminary immunohistochemical analysis of the von Willebrand factor (vWF) related antigen on sections from all experimental groups, and found that R-1mAb/R-2mAb treatment significantly suppressed vWF positive vessels. However, it was hard to accurately evaluate vWF positive cells because of difficulties in identifying the little slit vessels in the R-1mAb and R-2mAb combination treated group (data not shown). It has been reported that CD34 is a more sensitive marker than vWF related antigen.[4] CD31 was also shown to be a sensitive marker in EC.[48] Among these markers, it has been reported that CD34 expression may be decreased by VEGF.[49] We thus used CD31 expression in the current study.

We performed real time PCR analysis of CD31 gene expression to evaluate neovascularisation in the liver. Figure 4 demonstrates that CD31 gene expression was significantly increased in association with liver fibrosis development. Similar to fibrosis area, both R-1mAb and R-2mAb significantly suppressed CD31 gene expression compared with the control group ($p<0.01$). The inhibitory impact was more potent with R-2mAb treatment than that with R-1mAb treatment ($p<0.01$), and the combination treatment of both mAbs almost abolished neovascularisation in the liver. Noteworthy was the finding that suppression of angiogenesis by treatment with R-1mAb and R-2mAb was of a similar magnitude to that of inhibition of fibrosis areas.

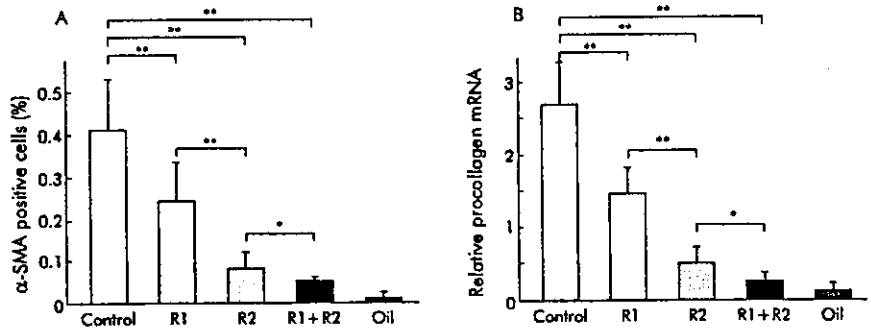


**Figure 6** Densitometric analysis of α smooth muscle actin (α-SMA) positive cells (A) and α1-(I)-procollagen mRNA expression (B) in the $CCl_4$ treated liver. α-SMA positive activated hepatic stellate cells and α1-(I)-procollagen mRNA were significantly reduced by R-1mAb and R-2mAb treatment. The inhibitory effect of R-2mAb was more potent than that of R-1mAb. The inhibitory effects of R-1mAb and R-2mAb on α-SMA and α1-(I)- procollagen expression exerted almost parallel reductions. Control, immunoglobin G treated mice (800 μg/mouse) (G1); R1, R2, R-1mAb and R-2mAb treated mice (800 μg/mouse) (G2 and G3, respectively); R1+R2, R-1mAb and R-2mAb combination treated group (G4); Oil, corn oil injected negative control mice. Data are means (SD) ($n=5$). $^*p<0.05$, $^{**}p<0.01$ between the indicated groups.

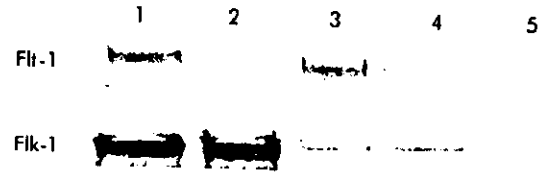


**Figure 7** Effects of R-1mAb and R-2mAb on the activation of vascular endothelial growth factor (VEGF) receptors VEGFR-1 (fms-like tyrosine kinase (Flt-1)) and VEGFR-2 (kinase-insert domain-containing receptor/fetal liver kinase-1 (Flk-1)). Fifteen minutes after injection of R-1mAb and R-2mAb, the liver was resected from three mice and pooled. The liver lysate was concentrated and used for immunoprecipitation, as described in the methods section. R-1mAb and R-2mAb significantly inhibited tyrosine phosphorylation of the respective receptors. Neither activation of VEGFR-1 nor that of VEGFR-2 was altered by administration of R-2mAb and R-1mAb, respectively. The activation level of VEGFR-1 was lower than that of VEGFR-2. Lane 1, immunoglobin G treated control group (G1); lane 2, R- 1mAb treated group (G2); lane 3, R-2mAb treated group (G3); lane 4, R-1mAb and R-2mAb combination treated group (G4); and lane 5, corn oil treated negative control group.

### Effects of R-1mAb and R-2mAb on HSC activation

It has been reported that not only SEC, but also activated HSC, express both VEGFR-1 and VEGFR-2.[23 24] Immunohistochemical examination showed that α-SMA positive cells were drastically reduced by treatment with R-1mAb and R-2mAb (fig 5A–5D). Computer assisted semiquantitative analysis showed that α-SMA positive cells in the R-1mAb and R-2mAb treated groups were significantly reduced compared with the control group ($p<0.01$) (fig 6A). We also performed real time PCR analysis to elucidate the effect of these mAbs on α1-(I)-procollagen mRNA expression. R-1mAb and R-2mAb also markedly suppressed mRNA expression of α1-(I)-procollagen in the liver compared with the control group ($p<0.01$) (fig 6B). The inhibitory effects of R-2mAb on both α-SMA positive cells and α1- (I)-procollagen mRNA expression were significantly stronger than those of R-1mAb ($p<0.01$). The inhibitory effects of R-1mAb and R-2mAb on α-SMA, α1-(I)-procollagen mRNA expression, and fibrosis area were almost identical, suggesting that suppression of HSC activation also contributed to the antifibrotic effect of these mAbs.

### VEGFR-1 and VEGFR-2 receptor activation in situ

To determine whether R-1mAb and R-2mAb at the dose used in the current study (800 μg/mouse) inhibited autophosphorylation in the liver, we investigated tyrosine phosphorylated VEGFR-1 and VEGFR-2 in the liver after intraperitoneally injection of R- 1mAb and R-2mAb. R-1mAb and R-2mAb significantly inhibited tyrosine phosphorylation of the respective receptors, and the combination treatment of R-1mAb and R-2mAb almost completely abolished phosphorylation of both receptors in the liver.

Neither activation of VEGFR-1 nor that of VEGFR-2 was altered by administration of R- 2mAb and R-1mAb, respectively (fig 7).

### Effect of VEGF on cultured activated HSC and SEC

It has been reported that HSC plated on collagen I are activated progressively whereas those on a basement membrane substratum resembling the normal subendothelial matrix of the liver (EHS matrix) remain quiescent.[9 10] We examined the effect of VEGF on HSC proliferation under different culture conditions. Figure 8A shows that both 10 and 100 ng/ml VEGF treatment did not increase in vitro proliferation on an EHS matrix whereas it was stimulated significantly on a collagen I coated dish ($p<0.05$), indicating that VEGF stimulated activated HSC proliferation but not quiescent HSC. We also examined whether VEGF increased synthesis of the extracellular matrix (ECM) component in activated HSC. As shown in fig 8B, VEGF treatment at a dose of 10 ng/ml significantly upregulated α1- (I)-procollagen mRNA synthesis in activated HSC.

We next examined the effect of VEGF on SEC proliferation. As it has been shown that VEGF is a survival factor for SEC,[45] we chronologically examined the proliferation assay of SEC in the presence of VEGF (10 ng/ml). Figure 8C reveals that proliferation of SEC was significantly increased on stimulation with VEGF over time. Without VEGF, these cells rapidly atrophied and died in a few days. We also examined whether VEGF induced production of factors by SEC that may have an impact on HSC biology, such as platelet derived growth factor and transforming growth factor β. We found that neither factor was increased by treatment with VEGF in SEC (data not shown). In addition to the MTT assay, we performed the [H]' incorporation experiment for in vitro proliferation of both HSC and SEC. Our results were similar to those of previously reported[51] MTT and [H]' incorporation assays (data not shown).

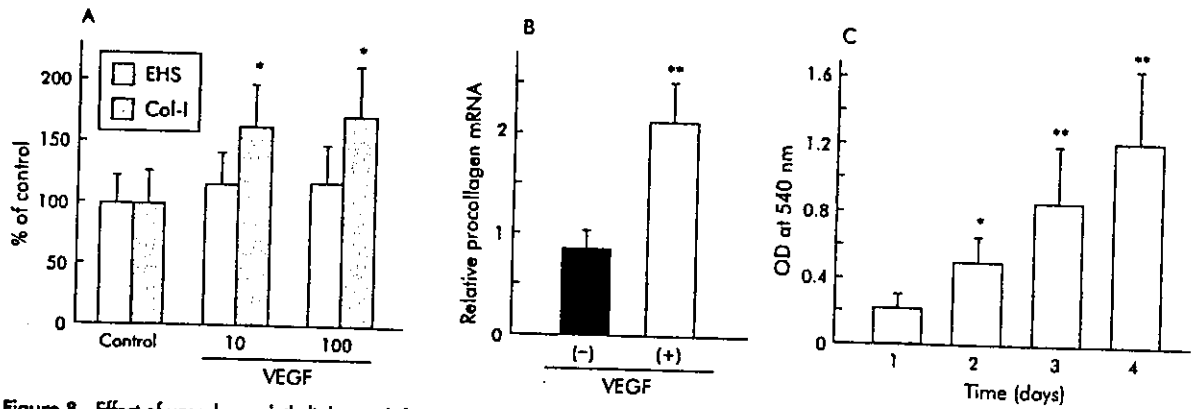


**Figure 8** Effect of vascular endothelial growth factor (VEGF) on proliferation and α1- (I)-procollagen mRNA expression of activated hepatic stellate cells (HSC) and hepatic sinusoidal endothelial cells (SEC) in vitro. Cell proliferation and mRNA expression were measured by the MTT assay and real time polymerase chain reaction, as described in the methods section, respectively. (A) At doses of 10 and 100 ng/ml, VEGF treatment did not increase the in vitro proliferation of HSC on an EHS matrix whereas it significantly stimulated proliferation on collagen I (Col-I). *$p<0.05$ compared with the control group. Control, untreated control group; VEGF, VEGF treated groups at doses of 10 and 100 ng/ml. (B) At a dose of 10 ng/ml, VEGF significantly increased α1-(I)-procollagen mRNA synthesis in activated HSC. **$p<0.01$ compared with the VEGF untreated group. (C) Proliferation of SEC was significantly increased over time on stimulation with VEGF (10 ng/ml). OD, optical density. *$p<0.05$, **$p<0.01$ compared with day 1. Data are means (SD) ($n=5$).

## DISCUSSION

Angiogenesis and fibrosis are key components in development, growth, wound healing, and regeneration. Recent studies have revealed that these processes commonly occur together in many disease states where neovascularisation is believed to initiate the pathological cascade.[4] Among the identified angiogenic factors to date, VEGF is one of the most potent and central factors in many physiological and pathological processes.[14-16] In liver fibrosis, it has been shown that VEGF expression increased in both human chronic liver diseases and experimental fibrogenesis.[10-12] It has also been reported that VEGF expression correlates with chronic liver disease associated angiogenesis and sinusoidal capillarisation.[12 32 33] We also observed that VEGF gene expression significantly increased during fibrosis development associated with neovascularisation in the liver, and that suppression of VEGF-receptor interaction significantly attenuated progression of liver fibrosis and angiogenesis.

The biological activities of VEGF are mediated mainly via two type III tyrosine kinase receptors—namely, VEGFR-1 and VEGFR-2—which serve different roles in angiogenesis and signal transduction pathways.[15 16 26 28] It has been reported that VEGFR-2 plays a more important role both in vitro and in vivo in several biological events.[15 16 35 36] Overexpression of VEGFR-2 in porcine EC caused actin reorganisation, chemotaxis, and mitogenesis in response to VEGF, although VEGFR-1 expression in the same cells had a minimal effect in vitro.[28] However, recent studies have revealed that VEGFR-1 is also involved in pathological angiogenesis, such as tumour growth.[30-34] In the present study, we found that inhibition of either VEGFR-1 or VEGFR-2 significantly attenuated liver fibrogenesis accompanied by angiogenesis suppression, and that treatment with R-2mAb was more potent than that with R-1mAb. The combination treatment with both mAbs almost completely attenuated liver fibrogenesis. These results indicate that VEGF-receptor interaction is a major regulator of the process of liver fibrosis. Both VEGFR-1 and VEGFR-2 were involved in liver fibrogenesis, and signalling through VEGFR-2 was a predominant pathway compared with that via VEGFR-1.

It is now recognised that activated HSC play an important role in liver fibrosis development.[1 34] In addition to EC, recent studies have shown that expression of VEGF and its receptor occurs in activated HSC.[22-24] This indicates that the cellular targets of VEGF are not confined to EC, and that VEGF responses reflect the combined effects on both EC and HSC. Recently, it has been shown that hypoxia induced VEGF expression was associated with angiogenesis and liver fibrogenesis. The authors showed that hepatic VEGFR-1 expression increased in liver fibrogenesis, which probably originated from activated HSC.[11] The in vitro study showed that VEGFR-1 was selectively increased in activated HSC under hypoxic conditions whereas expression of VEGFR-2 was not upregulated.[23] However, it should be noted that VEGFR-2 was constitutively expressed in activated HSC, and that the expression level of VEGFR-2 was much higher than that of VEGFR-1.[11 23 24] We found that activated α-SMA positive HSC and α1-(I)-procollagen mRNA were significantly suppressed by R-1mAb and R-2mAb. It has been reported that α-SMA expression in activated HSC may be downregulated by VEGF.[21] The authors also claimed that VEGF did not affect proliferation of activated HSC in vitro. In contrast, we and others have shown different results under different culture conditions,[24] suggesting that VEGF exerts a different biological effect on activated HSC under different culture conditions. After liver injury, HSC proliferate and become activated to the myofibroblastic phenotype, which is characterised by an increase in expression of α-SMA.[1] As it is now widely recognised that α-SMA is a useful and reliable marker for in vivo fibrogenesis,[1 38 40] we also employed this marker in the current study.

We also found that VEGF stimulated proliferation of activated HSC in vitro, indicating that VEGF-receptor interaction in HSC also plays an important role in liver fibrogenesis. We found that HSC did not respond to VEGF when they were cultured on Matrigel. Matrigel may sequester VEGF as it can bind with the proteoglycans of Matrigel. However, we found similar effects with low and high exogenous VEGF treatment (10 ng/ml and 100 ng/mL respectively). With high doses of VEGF, we assume that VEGF can act on HSC even if Matrigel may sequester some part of VEGF. A similar effect of VEGF on activated HSC in Matrigel has been reported.[24]

It would be important to elucidate the binding sites of R-1mAb and R-2mAb during liver fibrogenesis. We attempted to localise these binding sites of mAbs by immunohistochemical double staining but we failed to obtain good results. The background was very intense, and interpretation was very difficult (data not shown). Furthermore, the specific role of each receptor in activated HSC remains obscure at this time as we could not obtain specific R- 1mAb and R-2mAb for the rat. When mAbs against rat VEGFR-1 and VEGFR-2 become available, further studies may elucidate the role of each receptor in liver fibrogenesis.

VEGF was originally identified as a vascular permeability factor that increased microvessel permeability approximately 50 000 times more than histamine.[14 16 27] It induces extravasation of plasma proteins, leading to an increase in the ECM. It has also been reported that VEGF increased mRNA levels of connective tissue growth factor, which has known actions on ECM production.[35] In the current study, we found that VEGF significantly increased α1(I)-procollagen mRNA in activated HSC, indicating that VEGF stimulates liver fibrogenesis through both ECM production and proliferation in activated HSC. Nevertheless, it is well known that HSC are the primary ECM producing cell type but SEC also respond rapidly to injury by synthesising an isoform of the cellular fibronectin that stimulates HSC activation.[56] They also produce ECM components, such as type IV collagen and several proteoglycans.[57 58] In addition to its angiogenic activity, VEGF may also stimulate fibrosis development through these biological activities.

In summary, hepatic VEGF mRNA expression was significantly increased during the development of fibrosis, and both R-1mAb and R-2mAb treatment significantly attenuated the fibrogenesis associated with suppression of neovascularisation in the liver. The inhibitory effect of R-2mAb was more potent than that of R-1mAb, and the combination treatment with R-1mAb and R-2mAb almost completely attenuated liver fibrosis development. α-SMA positive activated HSC and α1-(I)-procollagen mRNA were significantly suppressed by R-1mAb and R-2mAb, and VEGF stimulated proliferation of activated HSC in vitro. These results suggest that interaction between VEGF and its receptors, which reflected the combined effects of both on HSC and SEC, was a prerequisite for liver fibrosis development.

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# Angiotensin-II induces the tissue inhibitor of metalloproteinases-1 through the protein kinase-C signaling pathway in rat liver fibrosis development

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**Abstract**

It has been shown that tissue inhibitor of metalloproteinases-1 (TIMP-1) plays an important role in the progression of liver fibrosis. TIMP-1 gene expression is regulated by several factors in vivo. Among them, angiotensin-II (AT-II) induces TIMP-1 in endothelial cells (EC) in vitro, however, the interaction between these molecules in liver fibrogenesis has not yet been elucidated. The aim of this study was to examine a possible association between TIMP-1 and AT-II both in vitro and in vivo using the clinically available angiotensin-I converting enzyme (ACE) inhibitor, perindopril (PE), and the AT-II type 1 receptor blocker (ARB), candesartan (CA). In cultured activated hepatic stellate cells (HSC), AT-II increased TIMP-1 in a dose- and time-dependent manner. CA and LY 333531, the protein kinase C (PKC) inhibitor, blocked this augmentation in a dose-dependent manner. In the $CCl_4$- and pig serum-induced rat liver fibrosis model, a clinically comparable low dose of PE and CA significantly suppressed liver fibrosis development associated with the suppression of TIMP-1 expression in the liver. AT-II induces TIMP-1 via type 1 receptor and PKC as an intracellular signaling pathway in activated HSC. These results suggested that the AT-II-induced TIMP-1 plays an important role in liver fibrosis development.


*Keywords:* Angiotensin-II; Hepatic stellate cells; Liver fibrosis; Protein kinase C; Tissue inhibitor of metalloproteinases-1

## 1. Introduction

Extensive remodeling of the extracellular matrix (ECM) has been shown to play a pivotal role during the development of liver fibrosis [1,2]. Gross remodeling of the ECM in the fibrotic liver is likely to be regulated by the net balance of synthesis and degradation of ECM. Alternation of the balance between matrix metalloproteinases (MMP) and their inhibitors, tissue inhibitors of metalloproteinases (TIMP), has been shown to play a key role in maintaining the balance between ECM deposition and degradation [3,4]. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a broad specific inhibitor of MMPs and functions by forming direct noncovalent 1:1 complexes with MMPs [5]. During liver fibrosis development, TIMP-1 expression in the liver TIMP-1 level was markedly up-regulated both in humans and murine fibrosis models [3,4,6–9]. The serum levels of TIMP-1 were increased in patients with chronic active liver disease, correlating with the histological degree of liver fibrosis [10]. We recently reported that TIMP-1 significantly promoted liver fibrosis development in a transgenic mouse model [11]. Moreover, in a rat model of reversible liver fibrosis, matrix remodeling and resolution of liver fibrosis were closely associated with a marked decrease in TIMP-1 expression [12].

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TIMP-1 gene expression was regulated by several factors in vivo [5]. It has been reported that factors, such as phobol esters, IL-1 β, and transforming growth factor-β (TGF-β), upregulated TIMP-1. It has been shown that angiotensin-II (AT-II), which is an octapeptide produced mainly by the proteolytic cleavage of its precursor angiotensin-I by angiotensin-I converting enzyme (ACE) in the renin-angiotensin system (RAS), also increased TIMP-1 gene expression in rat heart endothelial cells (EC) in vitro [13].

The RAS is a key mediator in the regulation of arterial blood pressure and body fluid homeostasis [14]. In the liver, it has been shown that the RAS is frequently activated in patients with chronic liver disease, such as cirrhosis [15,16]. AT-II has also been shown to induce contraction and proliferation of hepatic stellate cells (HSC), which play a pivotal role in liver fibrosis development [17]. It has been reported that inhibition of the RAS by the clinically used ACE inhibitor, perindopril (PE) and the AT-II type 1 receptor (AT1-R) blocker (ARB), candesartan (CA), significantly suppressed liver fibrosis development in several animal experimental models associated with the suppression of HSC activation [18–20].

Protein kinase C (PKC) is composed of a family of serine-thereonine kinases [21,22]. It has been postulated that activation of PKC is an important intracellular signaling pathway for several biological properties, such as cellular differentiation or growth [22,23]. It has been shown that AT-II stimulates TIMP-1 production by a PKC-dependent pathway in EC in vitro [13]. However, the interaction of these factors during liver fibrosis development has not been elucidated.

In the present study, we examined the interaction between AT-II and TIMP-1 in activated HSC. We also examined the signaling cascade, with a special focus on PKC. Furthermore, we elucidated the in vivo effect of RAS suppression with regard to TIMP-1 expression in rat liver fibrosis development.

## 2. Methods

### 2.1. Animal and compound

A total of 66 Male Fisher 344 rats, aged 6 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless-steel, mesh cages under control conditions of temperature (23±3 °C) and relative humidity (50±20%), with 10–15 air changes per hour and light illumination for 12 h a day. The animals were allowed access to food and tap water ad libitum throughout the acclimatization and experimental periods. ACE-I, PE, and ARB, CA, were supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan) and Takeda Pharmaceutical Co. (Tokyo, Japan), respectively. PKC specific inhibitor, LY333531 was supplied by Lilly Laboratories (Indianapolis, IN, USA) [24]. Pig serum and carbon tetrachloride ($CCl_4$) were purchased from Cosmo Bio. Co. (Tokyo, Japan) and Nacalai Tesuque. Co. (Kyoto, Japan), respectively.

### 2.2. Isolation and culture of hepatic stellate cell

The liver HSC were isolated from the liver of F344 rats as described previously [18]. The cells were plated at a density of $5 \times 10^5$ cells/ml on uncoated 60-mm plastic dishes. After 5 days in culture, HSC became myofibroblast-like with reduced lipid vesicles and increased immunoreactive α-SMA, and 7 days after plating, all of the cells were well-spread and α-SMA positive [25]. At day 10, activated HSC were arrested by serum-starvation for 24 h to avoid interference of the serum components. AT-II (1 μM) (Nacalai), CA (0.1 or 1 μM), and LY333531 (50 or 500 nM) were added for the respective studies.

### 2.3. The RNA expression of TIMP-1 mRNA by real-time PCR

TIMP-1 mRNA expression was evaluated by real-time PCR as described previously [26] for both the in vitro and in vivo studies. For the in vitro study, mRNA was extracted from HSC obtained form the untreated control, AT-II (1 μM)-treated and AT-II plus CA (1 μM)- and LY333531-treated groups (n = 4 each). For the in vivo study, half of the rats of each experimental group (n = 5) were used for measurement of hepatic TIMP-1 mRNA from the liver. For cDNA synthesis, Taqman reverse transcription reagents with oligo-dT primer were used as described in the manufacturer's manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA), which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). Relative quantification of gene expression was performed as described in the manual using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of target RNA as described for PE. The following temperature regimen was employed: holding 50 °C for 2 min, holding 60 °C for 30 min, holding 94 °C for 5 min, cycle 45 repeats at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. To prevent genomic DNA contamination, all RNA samples were subjected to DNase I digestion and checked by 40 cycles of PCR to confirm the absence of amplified DNA.

### 2.4. Animal treatment

In the current study, we employed two different rat liver fibrosis models with pig serum and $CCl_4$. The same

lot of pig-serum was used in all experiments as described previously [18]. The rats were randomly divided into seven groups. Groups 1–3 (G1- 3) and G4–G6 received 1 ml/kg per BW of $CCl_4$ and 0.5 ml of pig serum intraperitoneally twice weekly for 8 weeks, respectively. G1 and G4 were given the basal diet throughout the experiment, and were designed as a control group in the respective studies. The experimental animals in G2, G5 and G3, G6 received PE and CA by gavage once a day at the dose of 2 mg/kg, which is a clinically comparable dose for both compounds [18]. The rats in G7 received a phosphate buffer saline (PBS) injection instead of pig serum or $CCl_4$, and were given the basal diet. Groups of G1–G6 consisted of ten animals and G7 consisted of six rats, respectively. At the end of all experiments, all rats were sacrificed under ether anesthesia, and examined for the study items. The serum samples were obtained from the abdominal aorta. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

### 2.5. Histopathological examinations and several markers

Five-micrometer-thick sections of formalin-fixed and paraffin-embedded livers were processed routinely for Sirius-red (S-R) staining for the pig serum-treated groups and Azan-Marolly (A-M) for the $CCl_4$-treated groups, respectively. Semi-quantitative analysis of fibrosis development was carried out with the Fuji-BAS 2000 image analyzing system (Fuji, Tokyo, Japan) in six ocular fields (40 × magnification) per specimen of five rats as described previously [18]. The alanine aminotransferase aspartate (ALT) was assessed using the routine laboratory method, and the hepatic hydroxyproline content was determined as previously described [18].

### 2.6. Statistical analysis

To assess the statistical significance of inter-group differences in the quantitative data, Bonferroni's multiple comparison test was performed after one-way ANOVA, followed by Barlett's test to determine the homology of variance.

## 3. Results

### 3.1. Effect of AT-II on TIMP-1 mRNA expression in the activated HSC

We first examined the effect of AT-II on TIMP-1 expression in activated HSC. As shown in Fig. 1A, the experiments were carried out with several ranges of AT-II. TIMP-1 mRNA expression was elucidated at doses of $10^{-11}$–$10^{-6}$ M of AT-II, and we found that the optimal induction was obtained at $10^{-7}$ M. At the dose of $10^{-7}$ M, TIMP-1 mRNA expression was increased rapidly upon the addition of AT-II. The maximal augmentation was 3.8-fold higher at 2 h and then declined over 5 h (Fig. 1B).

### 3.2. Effect of ARB and PKC inhibitor on AT-II-induced TIMP-1 mRNA expression in HSC

To elucidate that the signaling cascade of AT-II-induced TIMP-1 upregulation, we examined the effect of the AT1-R blocker, CA, and the PKC inhibitor, LY333531, on TIMP-1 mRNA expression in cultured activated HSC. As shown in Fig. 2A, TIMP-1 mRNA upregulation by AT-II was abolished by CA in a dose-dependent manner. A similar inhibitory effect was observed after treatment with LY333531. We also examined the effect of AT-II in quiescent isolated HSC, and found no TIMP-1 mRNA alternation as a result of AT-II treatment (data not shown).

### 3.3. Effect of RAS inhibition on TIMP-1 expression in the liver

As shown in Table 1, densitometric analysis showed that the areas of fibrosis were markedly suppressed in PE-treated (G2, G5) and CA-treated (G3, G6) rats in both the $CCl_4$ and pig serum treatment experiments as compared with the control groups (G1, G4) ($P < 0.01$). No fibrosis development was found in the PBS-treated group (G7). The liver hydroxyproline content significantly decreased in the PE- and CA-treated rats. On the other hand, the serum level of ALT did not show any difference among the experimental groups (G1–G3 and G4–G6) suggesting that the suppressive effects of PE and CA were not due to the non-specific cytoprotective effect.

We next examined the in vivo interaction of TIMP-1 and AT-II during liver fibrosis development. As shown in Fig. 3, TIMP-1 mRNA was significantly suppressed by RAS inhibition both in $CCl_4$- and pig serum-induced liver fibrosis development as compared with the control groups ($P < 0.01$). PE and CA exerted mostly parallel reductions on the fibrosis areas and TIMP-1 mRNA expression.

## 4. Discussion

In the current study, we showed that TIMP-1 mRNA expression was significantly increased by AT-II in activated HSC in a time- and dose-dependent manner by AT1-R. Furthermore, the results suggested that it is likely that TIMP-1 induction by AT-II is mediated by PKC as an intracellular signaling pathway. Further-

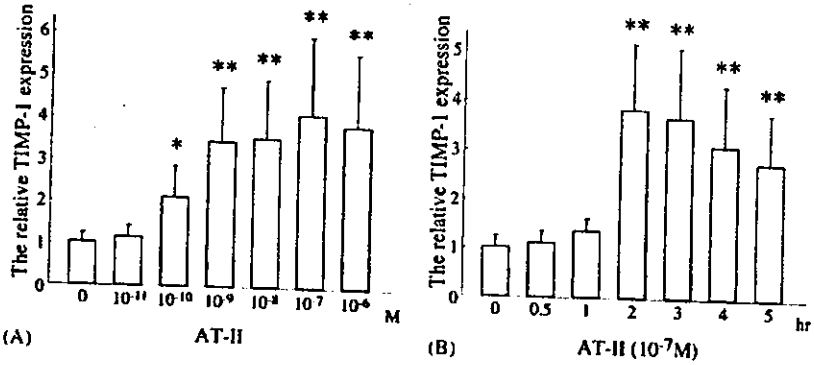


Fig. 1. Time course and dose-dependent studies of TIMP-1 mRNA induction by AT-II in activated HSC. TIMP-1 mRNA was measured by real-time PCR as described in Section 2. (A) TIMP-1 mRNA expression was examined at doses of $10^{-11}$–$10^{-6}$ M of AT-II. (B) TIMP-1 mRNA expression was increased rapidly upon the addition of AT-II. The data represent means ± S.D. (n = 5). *, **: Statistically significant difference compared with the AT-II-untreated control group ($P < 0.05$ and 0.01, respectively).

more, the suppression of AT-II attenuated liver fibrosis development in association with TIMP-1 inhibition.

To date, several types of AT-II receptors have been identified. Among them, AT1-R mediates most biological activities, such as intracellular $Ca^{2+}$ concentration, cell contraction, and proliferation [27]. In rat heart EC, TIMP-1 induction by AT-II is mediated by AT1-R [13]. We also found that AT-II-induced TIMP-1 up-regulation was attenuated by ARB, suggesting that AT1-R was also the main pathway of TIMP-1 induction by AT-II in activated HSC.

It is known that activated HSC is one of the main sources of TIMP-1 production in liver fibrogenesis [3,4]. It has been reported that RAS inhibition significantly suppressed the development of liver fibrosis accompanied by the inhibition of HSC activation [18–20]. In EC, TIMP-1 induction by AT-II depends on the PKC intracellular signaling pathway [13]. Similarly, we observed that PKC inhibition, attenuated AT-II-induced TIMP-1 mRNA upregulation. Although LY333531 has been shown to inhibit many types of PKC isoforms at the dose of 500 nM, the dose of 100 nM of this compound only inhibited the β-isoform of PKC in vitro [24,28]. We found that LY333531 had a sufficient inhibitory effect at the dose of 50 nM. It is likely that TIMP-1 induction by AT-II in activated HSC is mediated by the β-isoform of PKC. Taken together, it is possible that the effect of AT-II inhibition in liver fibrosis development was due to the suppression of TIMP-1 in activated HSC through the AT1-R and PKC-β isoform signaling cascade.

We did not find that AT-II induced TIMP-1 in quiescent HSC. Similarly, it has been reported that AT-II induced activated HSC contraction and proliferation, but not quiescent HSC [17]. The exact reason why AT-II acts only on activated HSC, but not on quiescent HSC is not clear at this time. It could be a result of either the machinery required for cell contraction or the absence of AT1-R in quiescent HSC. It has been shown that AT-II elicited a marked dose-dependent increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and cell contraction of activated HSC [17]. The increase in $[Ca^{2+}]_i$ is largely dependent on the entrance of $Ca^{2+}$ through L-type $Ca^{2+}$ channels [29]. It

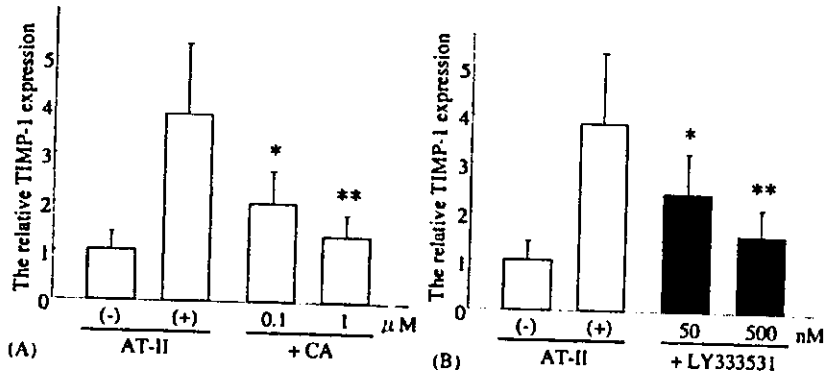


Fig. 2. Effect of ARB or PKC inhibitor on TIMP-1 mRNA expression in activated HSC. (A) TIMP-1 mRNA upregulation by AT-II was abolished by CA in a dose-dependent manner. (B) PKC inhibitor, LY333531 inhibited the TIMP-1 mRNA. LY333531 inhibits many types of PKC isoforms at the dose of 500 nM, whereas 50 nM of this compound only inhibits the β-isoform of PKC. AT-II (−): untreated control group, AT-II (+): AT-II (1 μM)-treated group. +CA and +LY333531: AT-II and CA (0.1 or 1 μM) and LY333531 (50 or 500 nM)-treated group, respectively. The data represent means ± S.D. (n = 4). *, **: Statistically significant difference compared with the AT-II (+) group ($P < 0.05$ and $P < 0.01$, respectively).

Table 1
Effect of RAS inhibitors on several markers in rats treated with $CCl_4$ and pig-serum

| Group | Treatment | Fibrosis area ($mm^2$/liver) | Hydroxyproline (μg/g) | ALT (U/l) |
|---|---|---|---|---|
| 1 | $CCl_4$ | 0.41±0.12[a] | 671.4±122.1 | 288.4±76.6 |
| 2 | +PE | 0.13±0.08[b] | 218.4±63.3 | 270.8±81.1 |
| 3 | +CA | 0.15±0.07[b] | 201.9±57.2 | 284.4±84.4 |
| 4 | Pig-serum | 0.24±0.04 | 382.2±88.6 | 61.8±11.4 |
| 5 | +PE | 0.08±0.02[c] | 89.7±16.3[c] | 64.2±10.9 |
| 6 | +CA | 0.09±0.02[c] | 86.9±15.9[c] | 63.6±13.8 |
| 7 | PBS | 0.02±0.01[c] | 24.2±10.4 | 60.2±12.4 |

[a] Data represents mean±S.D.
[b] Statistically significant different from group 1 and 4, respectively ($P < 0.01$).
[c] Statistically significant different from group 1 and 4, respectively ($P < 0.01$).

has been shown that there is a lack of these L-type $Ca^{2+}$ channels in quiescent HSC and their up-regulation after HSC activation [30]. These could be the reason underlying the former hypothesis. Further studies are required to elucidate the precise mechanism in the future.

TGF-β is an important cytokine in the regulation of the production of ECM proteins. It has been shown that TGF-β plays a pivotal role in liver fibrogenesis, and activated HSC is the main source of this cyokine [1]. We previously observed the effect of the AT-II inhibitors, PE and CA, on TGF-β expression in activated HSC in both in vitro and in vivo fibrogenesis [18]. Hepatic TGF-β protein and mRNA levels were significantly suppressed by treatment with PE and CA in pig serum-treated rats. AT-II increased the TGF-β mRNA, and this effect was totally blocked by CA in activated HSC in vitro. These results suggested that AT-II and AT1-R interaction plays an important role in regulating TGF-β expression in activated HSC.

The PKC family has been implicated in the regulation of a variety of biological actions, such as cellular differentiation, gene regulation, and proliferation [22,23]. We observed that immunostaining of the proliferating cell nuclear antigen (PCNA) of hepatocytes was not changed during liver fibrogenesis by treatment with PE and CA (data not shown). Previously, we also found similar results in a different choline-deficient amino-acid (CDAA) diet-induced liver fibrosis model [19]. These results suggest that the inhibitory effect of PE and CA was not due to the secondary effect of cell cycle regulation of hepatocytes, but also due to predominant action on activated HSC.

In summary, we showed that the ACE inhibitor, PE and ARB, CA at clinically realistic doses significantly reduced liver fibrogenesis by suppressing activated HSC with concomitant TIMP-1 down-regulation. TIMP-1 augmentation by AT-II was mediated through the AT1-R and PKC signaling pathway. Both agents are currently in wide use as anti-hypertensive agents over 100 countries without serious side effects. Considering the aspects of availability and safety, it may be worth considering the use of these agents for anti-fibrosis treatment through the suppression of TIMP-1.

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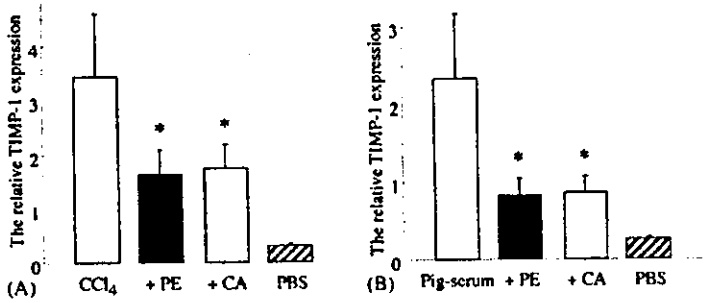


Fig. 3. TIMP-1 mRNA expression in the liver of $CCl_4$-treated (A) and pig serum-treated (B) rats. TIMP-1 mRNA was measured by real-time PCR as described in Section 2. The data represent mean±S.D. Each group consisted of five rats. *: Statistically significant difference compared with the control group ($P < 0.01$). $CCl_4$ and Pig-serum: $CCl_4$-treated (G1) and Pig-serum-treated (G4) control groups, PE: PE-treated rats, CA: CA-treated rats, PBS: PBS-treated rats (n = 5 in each group).

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# Improvement of fuel metabolism by nocturnal energy supplementation in patients with liver cirrhosis

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**Abstract**

*Aims*: patients with liver cirrhosis exhibit abnormal fuel metabolism, including increased fat and decreased glucose oxidation. Such altered energy metabolism is similar to that observed after starvation and could lead to malnutrition. We therefore studied whether nocturnal energy supplementation might improve the fuel metabolism in cirrhotic patients. *Methods*: 12 cirrhotic patients and 14 healthy controls participated in this study. Subjects in the two groups ate isonitrogenous (1.2 g/kg/day) and isocaloric (35 kcal/day) diets for 1 week before and during the study. On day 1 of the study, indirect calorimetry was carried out in the morning after an overnight fast. The next morning, the same measurement was performed after the patients took a liquid nutrient (Ensure Liquid®, 250 kcal) at 23:00 on day 1. Respiratory quotient (RQ), resting energy expenditure (REE), and substrate oxidation rates of glucose (% CHO), fat (% FAT) and protein were estimated from measured $VO_2$, $VCO_2$ and urinary nitrogen. *Results*: Significant decreases in RQ, and % CHO and a significant increase in % FAT were observed at baseline in cirrhotic patients as compared with controls. After the nocturnal energy supplementation, RQ, % CHO and % FAT in

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cirrhotic patients were significantly recovered, ending at levels close to normal. *Conclusions*: These results suggest that nocturnal energy supplementation could be useful to correct abnormal fuel metabolism and to prevent malnutrition in cirrhosis. 

*Keywords:* Nocturnal energy supplementation; Liver cirrhosis; Indirect calorimetry

## 1. Introduction

A significant proportion of patients with liver cirrhosis have protein-energy malnutrition [1,2]. Because poor nutritional state has been shown to lead to poor prognosis in cases of cirrhosis [2,3], it is important to adequately support the nutritional state of cirrhotic patients. As a characteristic of energy malnutrition, cirrhotic patients exhibit abnormal fuel metabolism, i.e. increased fat and decreased glucose oxidation [4,5]. This abnormal fuel metabolism could reduce the mass of adipose tissue and skeletal muscle, and subsequently worsen the malnutrition in cirrhosis. It has been reported that nocturnal energy supplementaton improves nitrogen balance in cirrhosis [6]. It may also have an effect on fuel metabolism as well, although little information on this question is currently available. In this study, we used indirect calorimetry to investigate whether or not nocturnal energy supplementation improved abnormal fuel metabolism in patients with cirrhosis.

## 2. Patients and methods

### 2.1. Patients

Twelve patients with liver cirrhosis (age $63 \pm 2$ years (mean $\pm$ SEM); male/female, 10/2) participated in this study. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histologic examination of liver biopsy specimens. Child–Pugh scores [7] of the patients were A in seven cases, B in two cases and C in three cases. Clinical profiles of the patients are shown in Table 1. Etiologies of cirrhosis were hepatitis C virus in 11 cases and alcohol in one case. Fourteen healthy volunteers served as controls (age, $55 \pm 3$ years; male/female, 11/3). Informed consent was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's review board for human research.

### 2.2. Methods

All subjects were hospitalized prior to the study. They ate isonitrogenous (1.2 g/kg/day) and isocaloric (35 kcal/kg/day) diets for one week before the study. The regular diet consisted of three meals: a breakfast at 08:00 h, a lunch at 12:00 h, and a dinner at 18:00 h. At 07:30 h on the first day of the study after an overnight fast,

indirect calorimetry was carrried out to estimate respiratory quotient (RQ), resting energy expenditure (REE), and substrate oxidation rates of glucose (% CHO), fat (% FAT) and protein (% PRO) from measured oxygen consumption per minute ($VO_2$), carbon dioxide production per min ($VCO_2$) and total urinary nitrogen (TUN) as previously described [2,5,8]. At 07:30 h on the second day, the same measurement was performed after each subject had taken 250 ml liquid nutrient (Ensure Liquid®, 250 kcal; energy composition, carbohydrate 54.5%, fat 31.5%, and protein 14.0%) at 23:00 h on the first day. The diet for the first and the second day was adjusted to give the subjects a total nitrogen and energy intake of 1.2 g/kg and 35 kcal/kg, respectively. TUN was determined by pyrochemiluminescence analyzer (PN-05, Mitsubishi Chemical, Tokyo, Japan). The nitrogen balance was calculated as the difference between the dietary nitrogen intake and TUN, in combination with the estimated fecal nitrogen loss and integumental nitrogen loss (4 g/day). The basal metabolic rate (BMR) was predicted by the Harris–Benedict formula [9].

### 2.3. Statistical analysis

Data were expressed as the mean and SEM. Differences in mean values between controls and cirrhotics, and changes in indirect calorimetric indices before and after the nocturnal energy supplementation were tested by one way analysis of variance followed by unpaired or paired $t$-test. Differences were considered significant when $P$ values were less than 0.05.

Table 1
Clinical profiles of the subjects[a]

| | Controls ($n = 14$) | Cirrhosis ($n = 12$) |
|---|---|---|
| Age (years) | 55 ± 3 | 63 ± 2 |
| Gender (male/female) | 11/3 | 10/2 |
| Height (cm) | 165 ± 3 | 160 ± 3 |
| Weight (kg) | 62 ± 3 | 61 ± 3 |
| Body mass index (kg/m$^2$) | 22.7 ± 0.5 | 23.7 ± 0.8 |
| Total-bilirubin (mg/dl) | 0.7 ± 0.1 | 1.8 ± 0.5* |
| Albumin (g/dl) | 4.4 ± 0.1 | 3.0 ± 0.2** |
| Alanine aminotransferase (IU/l) | 16 ± 2 | 64 ± 7** |
| Prothrombin time (%) | 94 ± 2 | 75 ± 6* |
| Creatinine (mg/dl) | 0.9 ± 0.1 | 0.9 ± 0.1 |
| Fasting plasma glucose (mg/dl) | 91 ± 4 | 99 ± 4 |
| Ammonia (μg/dl) | 59 ± 3 | 97 ± 12* |
| Free fatty acids (mEq/l) | 0.41 ± 0.05 | 0.56 ± 0.10 |

[a] Values are expressed as mean ± SEM.
* $P < 0.01$.
** $P < 0.001$ compared with controls.

Table 2
The effect of nocturnal energy supplementaion on metabolic parameters in cirrhosis[a]

| | Controls | Cirrhosis | |
|---|---|---|---|
| | | Before NES | After NES |
| RQ | 0.88 ± 0.01 | 0.81 ± 0.01 # | 0.86 ± 0.01* |
| REE/BMR | 0.98 ± 0.03 | 1.10 ± 0.04 | 1.04 ± 0.04 |
| % CHO | 55.4 ± 2.83 | 33.2 ± 4.2 # | 47.6 ± 4.6* |
| % FAT | 30.4 ± 2.5 | 54.7 ± 4.2 # | 38.7 ± 4.7* |
| % PRO | 14.2 ± 1.3 | 12.0 ± 1.4 | 13.7 ± 1.5 |

[a] Values are expressed as mean ± SEM. RQ, respiratory quotient; REE, resting energy expenditure; BMR, basal metabolic rate; % CHO, % FAT, % PRO, substrate oxidation rates for glucose, fat, and protein, respectively; NES, nocturnal energy supplementation.
# $P<0.001$ compared with controls.
* $P<0.05$ compared with cirrhosis before NES.

## 3. Results

Baseline indirect calorimetry on the first day of the study revealed significant decreases in RQ and % CHO and a significant increase in % FAT in cirrhotic patients as compared to controls ($P < 0.001$, respectively) (Table 2). There was no significant difference in REE/BMR or % PRO between cirrhotic patients and controls.

After the nocturnal energy supplementation, RQ, % CHO and % FAT were recovered significantly in cirrhotic patients ($P < 0.05$, respectively), and returned to nearly normal levels (Table 2). The nocturnal energy supplementation did not affect REE/BMR and % PRO significantly in cirrhotics (Table 2). Metabolic parameters were not altered by the nocturnal energy supplementation in control subjects (data not shown). There was no significant difference among cirrhotics in nitrogen balance before and after nocturnal energy supplementation (before 1.5 ± 0.7 g/day, after 0.8 ± 0.7 g/day, $P < 0.30$).

## 4. Discussion

It has been reported that the fasting or 24 h oxidation rate of glucose is decreased and that of fat is increased in patients with cirrhosis [2,4,5,10]. These alterations in substrate oxidation rates consequently reduce RQ in cirrhotics [2,4,5,10], as was shown also in this study. Decreased glucose oxidation could be due to the reduced glycogen storage in the liver and muscle [11], since the functional glycogen reserve of the liver and the muscle are decreased in cirrhosis. Glucose intolerance, which is observed in up to 80% of cases of cirrhosis [12], could also be a cause of this decreased glucose oxidation. As a result, fat or protein tissue is utilized as an alternate energy substrate.

This abnormal fuel metabolism is similar to the condition observed in normal subjects after 2–3 days of fasting [13]. In cirrhotic patients, even a single night of fasting could negatively effect the homeostasis in energy metabolism and induce the breakdown of endogenous fat or protein stores. A previous report has shown that limiting eating to two large meals induced a catabolic state in the morning both in cirrhotics and controls, and that more frequent meals (four to six times a day) improved this catabolism [14]. However, it is not usual for cirrhotics to occasionally lapse into taking only two meals a day, since about 90% of cirrhotic patients take three meals a day on a regular basis (Miwa Y, unpublished observation). It was also previously reported that a bedtime snack (50 g of carbohydrate) improved energy metabolism in cirrhosis [15]. Caution should be employed in giving additional energy intake to cirrhosis because it may induce obesity. Excess intake of energy could also lead to steatosis, which is contributory to fibrosis in chronic hepatitis C [16]. We have observed improved fuel metabolism with three regular meals plus nocturnal energy supplementaion without changing total energy and nitrogen intake. This result has confirmed that the frequency of meals rather than total amount of energy is important to improve abnormal fuel metabolism in cirrhosis.

In this study, improvement of nitrogen balance was not observed in cirrhosis. This might be due to the different efficacy of nutrient absorpion, because protein (mainly casein) in liquid nutrient could be more easily absorbed than protein in diet. As a whole, urinary nitrogen excretion might have increased substantially after the nocturnal energy supplementation, which could mask the improvement of nitrogen balance in this study. However, this possible increase of nitrogen loading did not induce clinical problems such as increase of blood ammonia level or deterioration of hepatic coma in the study.

We observed the improvement of RQ in five cases out of seven (71%) in child A, two out of three (67%) in child B, one out of two (50%) in child C. Although the number of the patients in this study was not enough to conclude, the nocturnal energy supplementation may not be beneficial for some patients with severe liver damage. We should therefore be careful about severity of liver disease to consider nocturnal energy supplementation as a therapeutic option for cirrhosis.

We used a liquid nutrient for this purpose for three reasons. First, the nutrient contained mainly carbohydrate (54.5%) as a substrate, which we expected would be adequate to compensate for the depleted glycogen stores in cirrhosis. Second, fat is also relatively rich (31.5%), which could be helpful to prevent the release of fatty acids for oxidaion from peripheral fat tissue. Third, compliance to the supplement was good in the study group. As a result of these factors, liquid nutrients have nearly normalized the dynamic parameters of fuel metabolism in cirrhosis. Improved nitrogen balance [4], normalized protein turnover, and decreased β-hydroxybutyrate [17] have all been previously reported as a result of nocturnal energy supplementation in cases of cirrhosis. Our present results suggest that nocturnal energy supplementation is also useful to correct the impaired fuel metabolism in cirrhosis. At least a portion of previous obsevations described above could be secondary to the corrected substrate oxidation rates after nocturnal energy supple-

mentation in cirrhotics. A long-term trial will be needed to determine whether or not nocturnal energy supplementation might actually prevent energy malnutrition and improve nutritional state index and prognosis in patients with cirrhosis.

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# Preoperative Administration of 5-FU and Interferon β May Prevent Recurrence of Hepatitis B and C Virus

TO THE EDITOR: Recurrence of viral hepatitis in liver transplantation is a major concern of the transplant hepatologist. Lamivudine and hepatitis B Ig for hepatitis B virus (HBV) and interferon and ribavuline for hepatitis C virus (HCV) have been tried (1–4). These clinical results of administration of these drugs in cases of liver transplantation have been satisfactory; however, high therapy costs are a major drawback to these treatments (5, 6).

We were involved with two cases that did not have HBV and HCV recurrence after living related liver transplantation (LRLT). The patients suffered from unresectable hepatocellular carcinoma and were treated with preoperative simultaneous administration of 5-fluorouracil (5-FU) (750–1000 mg) and interferon β (600 MU), for a total of nine times, and postoperative intraportal administration of 5-FU (150–250 mg *t.i.d.*), for a total of four times. The HCV patient has had no recurrence of HCV for 2 yr without any therapy. The HBV patient stopped hepatitis B Ig at 4 months after LRLT and has had no recurrence of HBV for 6 months with only lamivudine treatment. HBV DNA was negative and serum hepatitis B surface antibody titer was maintained above 200 U/ml. We have also performed a donor-specific transfusion via the portal vein after LRLT. Steroids were rapidly withdrawn within 1 month, and FK506 was rapidly reduced to 0.5 mg/4 days and 0.5 mg/14 days by 4 months after LRLT. It has been reported that combined therapy of interferon and 5-FU induces apoptosis of tumor cells or increases 5-FU cytotoxicity and inhibits hepatic carcinogenesis (7, 8). Preoperative elimination of HCV or HBV from the liver by interferon β and 5-FU might contribute to the prevention of the recurrence of viral hepatitis. Furthermore, rapid reduction of immunosuppression by intraportal donor-specific transfusion also might contribute to the defence of liver grafts against viruses (9, 10). In conclusion, although our experience regards only two cases, these findings may contribute to an understanding of the mechanism of recurrence and aid in the establishment of a defense against HBV or HCV with accompanying cost benefits.

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## Response to Dr. Ness *et al.*

TO THE EDITOR: We read with interest Ness and colleagues' article (1) on looking for possible predictors of inadequate colonic preparation for colonoscopy. Twenty-two percent of 649 patients were inadequately prepared and several independent predictors were described, including a later colonoscopy starting time; a reported failure to follow preparation instructions; inpatient status; indication of constipation; taking tricyclic antidepressants; male gender; and a history of cirrhosis, stroke, or dementia. In the same June, 2001 issue we reported on our experience with patient education programs before endoscopic procedures (2). We found a significant association between attendance in the education program and success of the endoscopy ($p = 0.0009$). Poor preparation that caused cancellations of procedures occurred in 26% of the patients who did not participate in the education program, compared to only 4% of patients who did ($p = 0.005$). This program also reduced the cost of colonoscopy by 9%. Thus, we would add the factor of patient education by an experienced nurse to those that predict an adequate bowel preparation for colonoscopy.

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## "Downhill" Esophageal Varices as an Iatrogenic Complication of Upper Extremity Hemodialysis Access

TO THE EDITOR: A case of "downhill" esophageal varices is presented. Downhill varices arise from shunting in cases of upper body (systemic) venous obstruction (1–8). They are less commonly observed than varices that arise from obstruction to portal venous flow (such as in cases of cirrhosis). Etiologies include lung or thyroid carcinoma, mediastinal fibrosis, superior vena cava (SVC) ligation, metastatic carcinoma, mediastinal mass of unknown origin, venulitis, and muscular constriction of abnormal extensions of posterior hypopharyngeal veins (2, 5–8).

A 42-yr-old African American female reported 4 months of intermittent nausea and abdominal discomfort and 3 months of melenic stools. Her history included pulmonary sarcoidosis, uveitis, and end-stage renal disease. Hemodialysis was initiated 23 yr earlier and was maintained via repeated use of upper extremity venous catheters. There was no history of jaundice, ascites, encephalopathy, or substance abuse. SVC stenosis was identified 4 yr earlier and recurred despite initial successful dilation. Physical examination revealed no neck or facial swelling and no signs of chronic liver disease. Engorged superficial veins were noted to run cephalocaudad along the chest and upper abdomen; however, no "caput medusae" were noted. Laboratory results were normal for liver-associated enzymes, tests of synthetic liver function, serum iron indices, and serum ceruloplasmin and were negative for chronic hepatitis B and C, primary biliary cirrhosis, and autoimmune hepatitis. Esophagogastroduodenoscopy unexpectedly revealed three columns of grade 2 varices in the mid- to distal esophagus with no stigmata of recent hemorrhage.

Abdominal magnetic resonance imaging (MRI)/magnetic resonance venogram (MRV) was obtained to evaluate the portal vasculature. The portal vein was normal and no portal venous collaterals were observed. The flow in the azygous vein was reversed, in the same direction as that in the aorta (downhill) (Fig. 1A). Contiguous downhill flow was also observed in distended subcutaneous veins of the abdominal and chest walls (Fig. 1B). SVC occlusion at a site closer to the heart than the insertion of the azygous system into the SVC was identified by venography, and an enlarged hemiazygous vein was seen to fill the inferior vena cava via collaterals. To date, the patient has not had clinical signs of SVC syndrome. Her esophageal varices were attributed to SVC obstruction. Sarcoidosis is not a likely etiology for her varices because of the lack of portal hypertension and the lack of signs of biliary cirrhosis (9).

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## Irregular Regeneration of Hepatocytes and Development of Hepatocellular Carcinoma in Primary Biliary Cirrhosis

TO THE EDITOR: Irregular regeneration of hepatocytes features anisocytosis and pleiomorphism of hepatocytes, nodularity and maplike distribution of hepatic parenchyma, bulging of regenerative hepatocytes, and regeneration of atypical hepatocytes. Recently, the observation of these conditions to a high degree in biopsied liver specimens has been reported to be a risk factor in the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis and cirrhosis of liver caused by hepatitis C virus infection (1, 2). We have previously reported in this journal (3) that it is not a rare event for HCC to arise from patients with primary biliary cirrhosis (PBC). However, the relationship between irregular regeneration of hepatocypes and the development of HCC in PBC patients has not been investigated. Here, we have studied prospectively whether irregular regeneration of hepatocytes is a risk factor for HCC in PBC patients. Liver biopsy specimens obtained from 40 PBC patients lacking in all known hepatitis viral markers were initially evaluated. The 40 specimens showed variations in staging according to Scheuer's classification (4): 15 were stage I, 12 were stage II, 10 were stage III, and three were stage IV. Severe irregular regeneration of hepatocytes was observed in five of the 40 PBC livers. All five specimens were of stage III or IV. Over the course of 5 yr of follow-up, four of these five patients developed HCC. In contrast, none of the remaining 35 PBC patients with mild or no irregular regeneration developed HCC. Although this finding needs to be confirmed by larger prospective studies, it is concluded that severe irregular regeneration of hepatocytes is a risk factor for the development of HCC in PBC patients.

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## Real-Time Measurement of Anti-HBs Level and Donor-Specific Transfusion via Portal Vein May Reduce Amount of HBIG After Living Related Liver Transplantation

TO THE EDITOR: The use of high doses of *i.v.* hepatitis B immune globulin (HBIG) after liver transplantation has reduced hepatitis B virus (HBV) recurrence and improved survival (1–3). The typical prophylactic regimen consists of high doses of *i.v.* HBIG (10,000 IU) during the nonhepatic phase, and subsequent doses of *i.v.* HBIG of 10,000 IU/day for the 1st wk and 10,000 IU/wk up to the 1st month. It is more effective to maintain an anti–hepatitis B surface (anti-HBs) titer above 500 U/ml for prevention of recurrence of HBV (3). Although the beneficial effect of long term HBIG administration has been widely accepted, the required expense for this therapy amounts to $27,000 for 1 wk, thereby placing a considerable financial burden on the patients. We have been involved with two cases of living related liver transplantation (LRLT) in HBV cirrhosis. In these cases, 100 mg/day of lamivudine was administered 4 wk before LRLT and 10,000 IU of HBIG was administered 2 consec-

utive days after LRLT. After this, the amount of HBIG was determined based on the real-time measurement of anti-HBs titer using lumipuls ƒ (Fuji Rebio, Tokyo, Japan), which takes only 1 h. The first patient took 2,000 U of HBIG per 4 days for maintaining over 500 U/ml and then 2,000 U/month was given 2 months after LRLT. One year after transplantation, HBIG was stopped and HBV vaccination (4) was administered. A second patient also underwent the same therapy regimen as the first; however, HBIG treatment was stopped in this patient after 5 months so as to be able to maintain anti-HBV titer above 200 U/ml without the administration of HBIG. Real-time measurement of anti-HBs titer might obviate HBIG treatment, which could spare the patient the cost of $7,000 in the 1st operative wk. We also have performed donor-specific transfusion via the portal vein after LRLT (5, 6). In these patients, steroid therapy was withdrawn within 1 month and FK506 rapidly reduced to 1 mg/day, and 0.5 mg/14 days by 4 months after LRLT. The rapid reduction of immunosuppressants might lead to success in the reduction of HBIG and the vaccination of HBV. In conclusion, *i.m.* HBIG combined with lamivudine or low dose HBIG combined with lamivudine have been demonstrated (7, 8); however, our clinical trial by intraportal donor-specific transfusion and further understanding of the immunological mechanism in LRLT might contribute to the reduction of HBIG and prevent the recurrence of HBV.

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# Clarithromycin Use Preceding Fulminant Hepatic Failure*

TO THE EDITOR: Idiosyncratic drug-induced fulminant hepatic failure (FHF) has been reported on one occasion with clarithromycin (Biaxin, Abbott Laboratories, North Chicago, IL) (1). We present a case of FHF associated with outpatient clarithromycin use in a patient with end-stage renal failure.

A 40-yr-old female presented with a 2-day history of weakness, nausea, abdominal pain, and fevers. Two weeks prior, the patient was perscribed a 7-day course of amoxicillin followed by a 7-day course of clarithromycin (500 mg *b.i.d.*) for an upper respiratory infection. The medical history was significant for end-stage mesangioproliferative glomerulonephritis on hemodialysis, peritonitis related to peritoneal dialysis, anemia, hypertension, isoniazid prophylaxis in 1975 for tuberculosis exposure, pancreatitis, and pulmonary sarcoidosis without steroid use. The surgical history was notable for a cholecystectomy, left upper extremity arteriovenous fistula, total abdominal hysterectomy with bilateral salpingoophrectomy, and an appendectomy. The outpatient medications included Renagel (Genzyme, Cambridge, MA), Epogen (Amgen, Thousand Oaks, CA), felodipene, quinine, doxazosin, Nephrocaps (Fleming, Fenton, MO), diphenhydramine, and minoxidil. She had no known drug allergies. There was no history of alcohol or acetaminophen use. On evaluation, the patient was febrile to 38.6°C with otherwise normal vital signs. Physical examination was notable for mild right upper quadrant abdominal tenderness without signs of chronic liver disease. Significant data included an absolute eosinophil count of 420; Hb, 9.3 g/dl; platelets, 154,000/μl; PT, 12.7 s; PTT, 35.3 s; total bilirubin, 0.6 mg/dl; AST, 17 U/L; ALP, 68 U/L; and ALT, 12 U/L. The patient was admitted to the hospital and treated with ampicillin/sulbactam.

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