Table 2 (*Continued*)

Age (years)	Healthy subjects					Cirrhotic patients			Estimated abnormal	
	n	25th	Median	75th	90th	25th	Median	75th	%	*n*
65–69	45	0.379	0.420	0.561	0.812	0.416	0.486	0.586	5.1	4/78
Total	542								14.2	40/281
RTT-C										
40–44	98	0.352	0.392	0.445	0.508	0.386	0.445	0.599	30.8	4/13
45–49	112	0.386	0.427	0.490	0.627	0.373	0.419	0.466	14.8	4/27
50–54	109	0.385	0.424	0.499	0.647	0.382	0.436	0.519	3.3	1/30
55–59	98	0.388	0.422	0.513	0.700	0.385	0.444	0.529	11.1	6/54
60–64	79	0.398	0.468	0.572	0.670	0.397	0.455	0.601	16.5	13/79
65–69	44	0.395	0.444	0.590	0.903	0.445	0.518	0.632	1.3	1/78
Total	540								10.3	29/281

between alcoholic and non-alcoholic cirrhosis patients. Aging also showed profound effects on the test results. Weissenborn et al. [14] also reported that aging has significant effects on the NCT-A and NCT-B results. Neuropsychological tests have been reported to show learning effects or effects of increasing familiarity with the tests as they are repeated [6,20]. However, we noted no such effects with our system because it was designed so that only a short practice is conducted before the main tests. The level of education is also expected to affect the test results, but this effect was thought to be minimal on our test system because our test system is relatively simple and more than 90% of the population in Japan receives education for at least 12 years. Some of the liver cirrhosis patients enrolled in our study were taking drugs for hyperammonemia, but these drugs had no effects on the test results and abnormalities could be detected by each test.

Abnormal values for the tests included in our test system were determined based on the results obtained from healthy adult volunteers. The cut-off values were set at the 10th/90th percentiles, which are statistical outliers in healthy subjects, so that abnormal values were not overestimated. The incidence of abnormalities determined in liver cirrhosis based on these cut-off values was about 25% for each test, and 58.2% of the 292 liver cirrhosis patients showed deviations from the 10th/90th percentile cut-off values for at least 1 test. These percentages are lower than the 30–84% prevalence of SHE reported in liver cirrhosis patients [1–14]. Because our data were obtained from a large number of subjects in a multicenter clinical trial, however, the 10th/90th percentile cut-off values determined by us are expected to provide a simple method to screen SHE patients from liver cirrhosis patients. SHE is suspected if the results of any of the eight tests included in our test system are abnormal, but further studies are needed to determine if the diagnosis of SHE should be made only when two or more tests give abnormal values.

In general, SHE is diagnosed based on the results of multiple tests designed to assess performance cognition. Our test system also assesses multiple performance cognition functions (psychomotor function, attention, memory, and special cognition function) based on the results of eight tests. At present, there is no method in which test results are scored to make a diagnosis of SHE based on overall assessment of the test results. We were also unable to draw any definite conclusion from the scoring of the test results. Our results showed, however, that many liver cirrhosis patients who show abnormality in one test also show abnormality in other tests. In the future, it will be necessary to weight each test before scoring the abnormalities and making an overall assessment of the test results.

Recently, 11 types of neuropsychological tests conducted by Weissenborn et al. [14] and the CFF test [16] showed that low-grade HE could be diagnosed with great sensitivity and specificity, but their SHE extraction rate was similar to that obtained by us, and we do not believe that the diagnostic sensitivity can be enhanced by increasing the number of tests conducted.

Because the test results are affected by a number of factors such as the subject's physical condition, fatigue, sleeping time, experience operating computers, ocular disease, and testing environment, it is recommended that these factors be eliminated as much as possible when using our test system.

At present, there is no standard diagnostic method or criteria for SHE in Japan. The computer-aided quantitative neuropsychological test system developed by us converted two- or three-dimensional tests using paper and blocks into two-dimensional tests that can be conducted using a touch panel so that SHE can be easily diagnosed in daily clinical practice. Because the main objective of the present study was to computerize the tests commonly conducted to diagnose SHE, the test system developed by us was not compared to the conventional tests using paper and blocks. Further studies are needed to determine if our test system is superior to the conventional test methods.

Further prospective studies are also needed to determine, using the test system developed by us, the percentage of patients in whom SHE progresses to clinical hepatic encephalopathy by conventional coma grade, and determine the effects of SHE on quality of life by SF-36 (Short form 36) and the relationship between SHE and etiology of liver cirrhosis.

Table 2
Estimated abnormal rate of cut-off value (10th/90th percentile; healthy subjects) and standard value by NP-test (the data represents medians, with interquartile ranges)

Age (years)	Healthy subjects					Cirrhotic patients			Estimated abnormal	
	n	25th	Median	75th	90th	25th	Median	75th	%	*n*
NCT-A										
40–44	98	20.7	24.8	29.3	36.2	25.2	31.4	42.7	35.7	5/14
45–49	112	23.2	27.3	34.4	41.7	26.7	31.2	34.6	3.9	1/26
50–54	109	25.8	31.4	35.6	41.4	33.6	40.7	51.3	42.4	14/33
55–59	98	28.4	33.4	42.5	51.0	33.2	38.9	45.5	16.7	9/54
60–64	78	32.6	37.5	44.3	55.8	36.1	46.0	52.5	17.3	14/81
65–69	45	33.7	45.1	54.4	62.1	36.6	45.0	56.8	14.3	11/77
Total	540								18.9	54/285
NCT-B										
40–44	98	30.4	37.5	45.3	64.1	36.8	53.2	59.8	21.4	3/14
45–49	112	35.3	43.5	54.7	72.7	46.6	54.0	65.7	16.0	4/25
50–54	109	41.4	50.4	62.4	76.1	61.2	75.9	93.7	46.9	15/32
55–59	95	49.3	59.2	74.8	101.0	54.7	74.9	104.8	28.3	15/53
60–64	78	55.0	67.4	102.5	129.1	65.1	90.0	114.5	16.7	13/78
65–69	43	61.4	80.5	121.1	142.4	74.7	88.7	117.5	9.2	6/65
Total	535								21.0	56/267
FPT										
40–44	98	1.3	1.6	2.1	2.4	1.6	2.7	3.5	57.1	8/14
45–49	112	1.4	1.8	2.2	3.0	1.6	2.0	2.6	19.2	5/26
50–54	108	1.4	1.9	2.4	3.7	1.8	2.3	2.8	15.2	5/33
55–59	96	1.5	1.9	2.7	3.7	1.9	2.2	2.8	11.5	6/52
60–64	77	1.7	2.2	2.9	3.8	1.9	2.7	3.4	22.1	17/77
65–69	41	1.8	2.5	3.8	6.2	2.4	3.1	4.6	9.9	7/71
Total	532								17.6	48/273
DST										
40–44	96	20.0	25.0	27.0	31.0	17.0	21.0	28.8	30.8	4/13
45–49	110	17.0	22.0	25.0	29.0	20.5	22.0	24.5	4.2	1/24
50–54	105	16.0	19.8	22.0	26.0	14.0	16.5	19.5	43.8	14/32
55–59	91	13.0	16.3	20.0	23.0	13.0	17.0	21.0	21.7	10/46
60–64	74	11.9	14.0	17.0	21.0	12.0	15.0	19.0	23.9	16/67
65–69	41	9.6	12.0	15.0	18.0	12.0	14.0	17.0	1.6	1/64
Total	517								18.7	46/246
BDT										
40–44	98	9.1	10.6	12.4	14.9	10.9	16.0	17.9	57.1	8/14
45–49	112	10.3	12.9	16.0	18.6	12.3	14.4	17.5	22.2	6/27
50–54	109	11.3	13.9	16.7	19.7	12.5	15.6	20.0	27.3	9/33
55–59	99	11.9	14.6	20.7	27.1	13.4	17.8	23.5	11.1	6/54
60–64	79	13.9	17.5	21.5	25.8	15.2	20.6	27.3	25.6	21/82
65–69	45	15.7	20.3	24.8	[28.5	19.5	23.9	28.8	27.5	22/80
Total	542								24.8	72/290
RTT-A										
40–44	98	0.300	0.331	0.373	0.427	0.312	0.379	0.436	25.0	3/12
45–49	111	0.302	0.353	0.402	0.487	0.316	0.410	0.479	22.2	6/27
50–54	109	0.328	0.372	0.434	0.521	0.332	0.394	0.529	30.0	9/30
55–59	99	0.331	0.367	0.471	0.623	0.361	0.408	0.508	11.1	6/54
60–64	79	0.348	0.412	0.502	0.639	0.381	0.497	0.625	21.3	17/80
65–69	45	0.348	0.417	0.635	0.875	0.435	0.565	0.718	10.3	8/78
Total	541								17.4	49/281
RTT-B										
40–44	98	0.331	0.363	0.393	0.432	0.352	0.394	0.440	30.8	4/13
45–49	112	0.333	0.365	0.402	0.474	0.361	0.389	0.410	18.5	5/27
50–54	109	0.344	0.375	0.420	0.465	0.352	0.385	0.442	23.3	7/30
55–59	99	0.349	0.385	0.448	0.556	0.356	0.392	0.436	9.3	5/54
60–64	79	0.363	0.394	0.445	0.568	0.367	0.437	0.520	19.0	15/79

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Angiotensin-II Type 1 Receptor Interaction Is a Major Regulator for Liver Fibrosis Development in Rats

HITOSHI YOSHIJI, SHIGEKI KURIYAMA, JUNICHI YOSHII, YASUHIDE IKENAKA, RYUICHI NOGUCHI, TOSHIYA NAKATANI, HIROHISA TSUJINOUE, AND HIROSHI FUKUI

The renin-angiotensin system (RAS) is frequently activated in patients with chronic liver diseases. Angiotensin-II (AT-II) has been suggested to play an important role in liver fibrogenesis. It induces hepatic stellate cell (HSC) proliferation and up-regulates the transforming growth factor β_1 (TGF-β_1) expression via AT-II type 1 receptor (AT_1-R) *in vitro*. The aim of the present study was to examine the *in vivo* effect of candesartan (CA), a clinically used AT_1-R blocker (ARB), and perindopril (PE), an angiotensin-converting enzyme (ACE) inhibitor (ACE-I), on pig serum-induced liver fibrosis development in rats. The clinically available comparable doses of CA and PE significantly attenuated the fibrosis development. These inhibitory effects of PE and CA were also found in the on-going liver fibrosis model. The hepatic hydroxyproline and serum fibrosis markers were significantly suppressed by CA and PE treatment. Furthermore, the α smooth muscle actin (α-SMA) positive cells in number were markedly suppressed by CA and PE treatment. Similarly, the hepatic TGF-β_1 protein and messenger RNA (mRNA) levels were significantly suppressed. Our *in vitro* study showed that AT-II increased the TGF-β_1 mRNA expression in the activated HSCs, and this effect was totally blocked by CA. These results suggested that the RAS, especially AT-II and AT_1-R interaction plays a pivotal role in liver fibrosis development through HSC activation. Because both CA and PE are widely used in clinical practice without serious side effects, these drugs may provide an effective new strategy for anti–liver fibrosis therapy. (HEPATOLOGY 2001; 34:745-750.)

The renin-angiotensin system (RAS) is reportedly activated in patients with chronic liver diseases, such as cirrhosis.[1-3] Angiotensin-II (AT-II), which is an octapeptide produced mainly by proteolytic cleavage of its precursor AT-I by angiotensin-converting enzyme (ACE), has many physiologic effects, including vascular hormonal secretion, tissue growth, and neuronal activities.[4,5] AT-II is also considered a potential mediator of intrahepatic portal hypertension, because its plasma level was increased in patients with cirrhosis, and its administration induced elevation of the portal pressure.[6,7] To date, several types of AT-II receptors have been identified.[5] The AT-II type 1 receptor (AT_1-R) mediates most of the biological effects of AT-II, including increase in the intracellular Ca^2 concentration, cell contraction, and proliferation.[5] Recently, losartan, which is used clinically as an AT_1-R antagonist, has been shown to reduce the portal pressure in hepatic cirrhosis.[8] It has been revealed that AT-II induced contraction and proliferation of hepatic stellate cells (HSCs).[9] AT-II also increased the transforming growth factor β_1 (TGF-β_1) and collagen-I gene expression in the fibroblasts *in vitro*.[10-12] These biological actions were mediated predominantly through AT_1-R. Accordingly, AT-II and AT_1-R interaction seems to have a pivotal role in liver fibrosis development. Evidence that these molecules are important *in vivo* in the development of hepatic fibrosis, however, is not available.

In the present study, we examined the effect of inhibiting the RAS, especially the AT-II and AT_1-R interaction, on liver fibrosis induced by administration of pig serum. We used clinically available compounds: an ACE inhibitor (ACE-I) and an AT_1-R blocker (ARB) at therapeutic concentrations. We also examined the effect of these agents on HSC activation and the hepatic TGF-β_1 level during liver fibrosis development.

MATERIALS AND METHODS

Animals. Male Fischer 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°C ± 3) and relative humidity (50% ± 20%), with 10 to 15 air changes per hour and light illumination for 12 hours a day. After a 1-week acclimatization period, the rats were divided into 4 experimental groups. The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods.

Compounds and Animal Treatment. The ACE-I, perindopril (PE), and ARB, and candesartan (CA) were supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan) and Takeda Pharmaceutical Co. (Tokyo, Japan), respectively. The pig serum was purchased from Cosmo Bio. Co. (Tokyo, Japan). The same lot of serum was used in all experiments. The rats were randomly divided into 6 groups. Groups 1 to 5 (G1-5) received 0.5 mL of pig serum intraperitoneally twice weekly for 8 weeks. G1 was given the basal diet throughout the experiment, and was designed as a control group. One week after the pig serum injection, the experimental animals in G2 and 3 started to receive PE

Abbreviations: RAS, renin-angiotensin system; AT-II, angiotensin-II; ACE-I, angiotensin-converting enzyme inhibitor; AT_1-R, angiotensin-II type 1 receptor; HSCs, hepatic stellate cells; TGF-β_1, transforming growth factor β_1; ARB, angiotensin-II type 1 receptor blocker; PE, perindopril; CA, candesartan; PBS, phosphate-buffered saline; α-SMA, α-smooth muscle actin; ALT, alanine transaminase; T Bil, total bilirubin; P-III-P, procollagen III-N-peptide; mRNA, messenger RNA; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor.

From the Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan.

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S.K.'s present address is at the Third Department of Internal Medicine, Kagawa Medical University, Kagawa, Japan.

Address reprint requests to: Hitoshi Yoshiji, M.D., Ph.D., Third Department of Internal Medicine, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan. E-mail: yoshijih@naramed-u.ac.jp; fax: (81)-744-24-7122.

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and CA by gavage once a day at a dose of 8 mg/kg, which is a clinically comparable dose for both compounds.[13,14] We also examined the effect of PE and CA on the on-going fibrosis model. Four weeks after the pig serum injection, the animals in G4 and G5 started to receive PE and CA in the same way as G2 and G3. The rats in G6 received a phosphate-buffered saline (PBS) injection instead of the pig serum and were given the basal diet. At the end of all the experiments, all rats were killed under ether anesthesia, and examined for the study items. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

Histopathologic and Immunohistochemical Examinations. Five-micrometer-thick sections of formalin-fixed and paraffin-embedded livers were processed routinely for hematoxylin-eosin and Sirius-red staining.[15] Immunohistochemical staining of α smooth muscle antigen (α-SMA) was performed as previously described with paraffin-embedded sections.[16,17] Briefly, formalin-fixed tissue sections were deparaffinized using xylene and rehydrated by passing through 99%, 95%, 70%, 50%, and 30% ethanol. Nonspecific antibody binding was blocked with serum diluted 1:50 in PBS, and the endogenous biotin was blocked with 0.1% avidin for 15 minutes, followed by 0.01% biotin for 15 minutes. These sections were washed once with PBS after each 15-minute incubation period, then reacted with a primary anti–α-SMA antibody (DAKO, Kyoto, Japan; 1:100) for 1 hour at room temperature. After washing twice with PBS, the sections were incubated with a second biotin-labeled rabbit anti-rat IgG (Novocastra, Burlingame, CA). The endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS for 30 minutes, followed by 5-minute washing with PBS. The sections were then incubated with conjugated streptavidin for 30 minutes, rinsed again in PBS, and finally incubated with diaminobenzidine for 3 minutes. Semi-quantitative analyses of fibrosis development and immunopositive cell area were carried out with Fuji-BAS 2000 image analyzing system (Fuji, Tokyo, Japan) in 6 ocular fields (magnification ×40) per specimen of 7 rats. We did not count the α-SMA–positive vessels in the portal area, which were assumed to be hepatic arteries. We only included α-SMA–positive cells in the sinusoidal lining for image analysis.

Serum Markers. At the end of the experiment, serum samples were obtained from the abdominal aorta. The alanine aminotransferase (ALT) and total bilirubin (T Bil) were assessed using the routine laboratory method. The serum markers for fibrosis development namely,7S-collagen, procollagen III-N-peptide (P-III-P), and hyaluronic acid were also measured as previously described.[18]

Hepatic Hydroxyproline Content and TGF-β_1 Expression. The hepatic hydroxyproline content was determined as previously described.[19] Briefly, liver specimens were weighed, and 20 mg of frozen samples were hydrolyzed in 6 mol/L HCl in an autoclave for 24 hours. After centrifugation, the supernatant was mixed with 1% phenolphthalein and 8N-KOH to obtain a solution at pH 7 to 8. This solution was stirred with KCl and borate buffer (pH 8.2) for 15 minutes at room temperature and for another 15 minutes at 0°C, after chloramine T solution was added and stirred for 60 minutes at 0°C. After addition of 3.6 mol/L sodium thiosulfate, the solution was incubated for 30 minutes at 120°C and stirred with toluene for 20 minutes. Then, Ehrlich's solution was added to the supernatant after centrifugation at 2,000 rpm at 4°C and left for 30 minutes at room temperature. The absorbance was measured at 560 nm. The hydroxyproline content was expressed as μg/g wet liver. The TGF-β_1 expression level in the liver was measured by the PREDICTA assay kit (Genzyme Co., Cambridge, MA). Liver lysates were prepared as described previously,[20] and the samples were equalized for protein concentration prior to measuring the TGF-β_1 level. All TGF-β_1 protein levels were assessed as the active form by addition of HCl[21] and were expressed as ng/mg of the total liver protein.

Isolation and Culture of HSCs. The liver HSCs were isolated from the liver of F344 rats as described previously,[22] with a minor modification. Briefly, the liver was perfused with Krebs'-Ringer solution followed by 0.1% pronase E and 0.032% collagenase (Nakarai, Kyoto, Japan) solution at 37°C. The digested liver was excised and minced, and incubated in Krebs'-Ringer solution containing 0.08% pronase E, 0.04% collagenase, and 20 μL/mL DNase for 30 minutes at 37°C (pH 7.3). After passage through a nylon mesh, the cells were centrifuged at 450g for 8 minutes. The HSC-enriched fraction was obtained by centrifugation in 8.2% Nycodenz (Nycomed Pharma AS, Oslo, Norway) solution at 1,400g for 20 minutes. The HSCs in the upper white layer were washed by centrifugation at 450g for 8 minutes and suspended in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 mU/mL streptomycin. The cell viability was over 95% as determined by the trypan blue exclusion test. The cells were plated at a density of 5×10^5 cells/mL on uncoated 60-mm plastic dishes. After 5 days in culture, HSCs 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.[23] From day 10, the medium with or without treatment of AT-II (1 μmol/L) (Nakalai, Kyoto, Japan) and CA (1 μmol/L) was changed every 24 hours and cell culture was continued until day 12.

RNA Expression of TGF-β_1 by Real-Time Polymerase Chain Reaction. TGF-β_1 messenger RNA (mRNA) expression was evaluated by real-time polymerase chain reaction (PCR) as described previously[23-25] for both the *in vitro* and *in vivo* studies. For the *in vivo* study, mRNA was extracted from the whole liver of each experimental group (n = 5). For the *in vitro* study, mRNA was extracted from HSCs of the untreated control, AT-II (1 μmol/L)-treated and AT-II plus CA (1 μmol/L)-treated groups (n = 4 each). For complementary DNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer's manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), 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 by using glyceraldehyde-3-phosphate dehydrogenase as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of the target RNA as described for PE. The following temperatures were used: hold 50°C for 2 minutes, hold 60°C for 30 minutes, hold 94°C for 5 minutes, cycle 45 repeats 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute. 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.

Statistical Analysis. To assess the statistical significance of intergroup 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.

RESULTS

Histologic Findings and Fibrosis Markers. As shown in Fig. 1A, Sirius-red staining showed that 8-week treatment with the pig serum resulted in a marked liver fibrosis development (G1). In contrast, treatment with PE (G2) and CA (G3) significantly attenuated the fibrosis development (Fig. 1B and C, respectively) compared with G1. Densitometric analysis showed that the fibrosis areas were markedly suppressed in the PE- and CA-treated rats ($P < .01$). We next examined whether these inhibitory effects could also be found in the ongoing fibrosis experimental model. In this experiment, PE and CA were administered 4 weeks after the pig serum injection. As shown in Fig.1D and E, both PE (G4, 1D) and CA (G5, 1E) exerted significant inhibitory effects on liver fibrosis development even in the ongoing fibrosis model. The degrees of inhibitory effects in G4 and G5 were similar to G2 and G3, respectively ($P < .01$ vs. control) (Fig. 2). No fibrosis development was found in the PBS-treated group (G6). The liver hydroxyproline content and the serum concentrations of 7-S collagen, P-III-P, and hyaluronic acid were all significantly

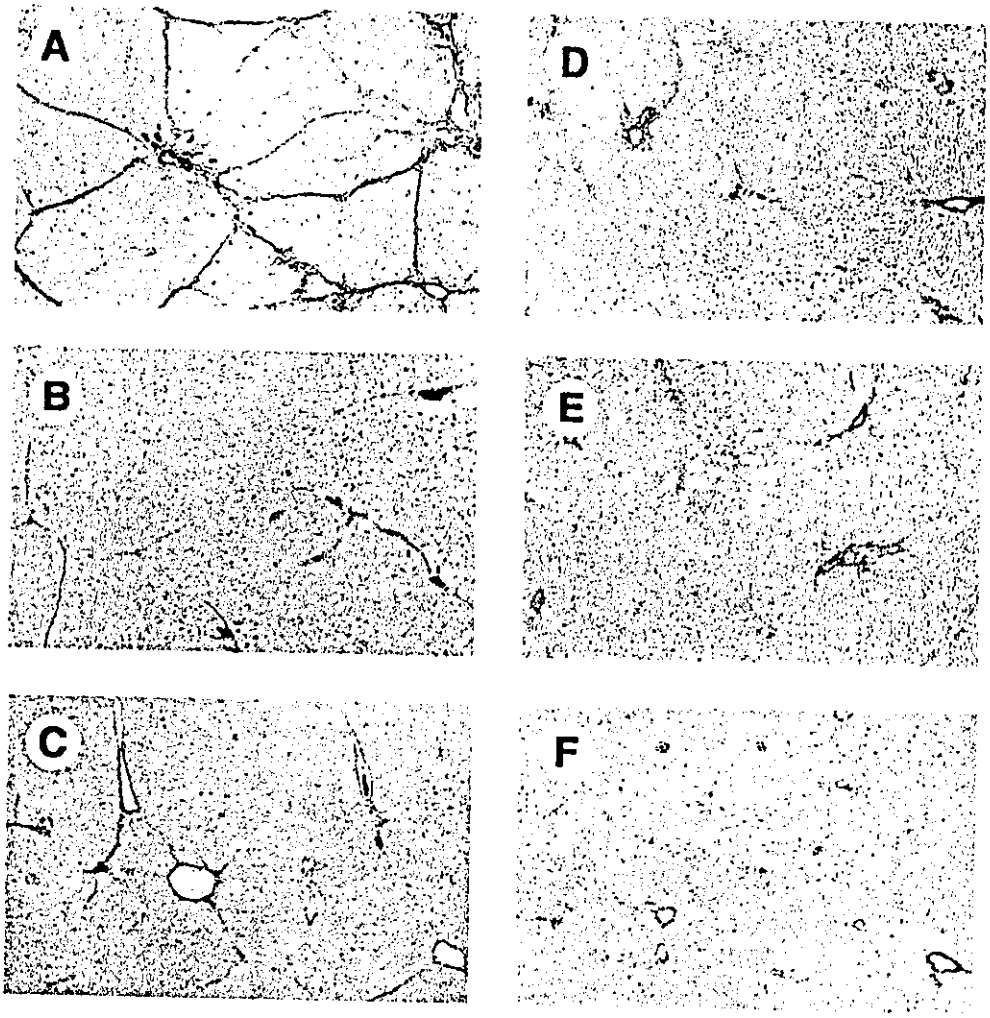

FIG. 1. Microphotographs of liver sections from the pig serum-treated rats. (A) Untreated control group (G1); (B and D) ACE-I (PE; 8 mg/kg/d) administration started 1 (G2) or 4 (G4) weeks after the pig serum injection. (C and E) ARB (CA; 8 mg/kg/d) administration started 1 (G3) or 4 (G5) weeks after the pig serum injection. The liver in G1 shows an extensive fibrosis development, but mild fibrosis in G2-5. (F) No fibrosis development was found in the PBS-treated group (G6) (Sirius-red staining, original magnification ×40).

decreased in the PE (G2)- and CA (G3)-treated rats, whereas the serum level of ALT and T Bil did not show any difference among all groups (Table 1). The body weights and liver weights did not show any significant differences among the groups, either (data not shown).

Immunohistochemistry. Immunohistochemical analysis of α-SMA was performed to examine the effect of these RAS-inhibitory agents on HSC activation during the fibrosis development. The activated HSCs, which express α-SMA and are therefore named myofibroblast-like cells, were significantly increased in the pig serum-injected group (G1, Fig. 3A). Treatment with PE (G2) and CA (G3) drastically reduced these positive cells in the liver (Fig. 3B and C, respectively). Computer-assisted semiquantitative analysis showed that the α-SMA–positive cells in the PE- and CA-treated groups were significantly reduced, compared with the control group ($P < .01$). Strong immunopositive staining was observed in the fibrotic septa (Fig. 3D). No α-SMA–positive cells in the sinusoidal wall could be found in the PBS-treated group (G6) (Fig. 4A). The inhibitory effects of PE and CA on the α-SMA–positive cells were of similar magnitude to the suppression of the fibrosis area development in the liver. These results suggested that prevention of fibrogenesis by PE and CA appeared to be related to the suppression of HSC activation and proliferation.

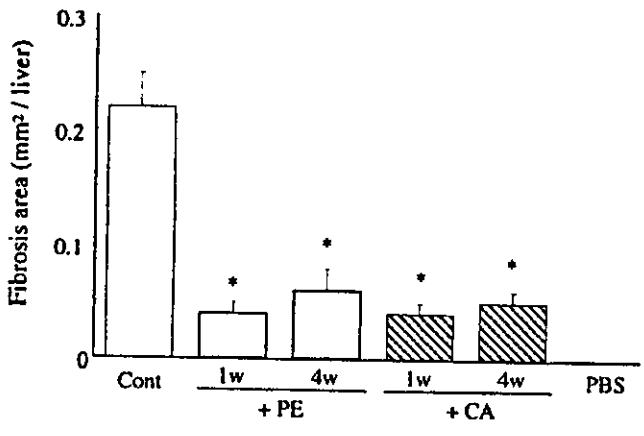

FIG. 2. Densitometric analysis of liver fibrosis development in the liver. ACE-I (PE; 8 mg/kg/d) administration started 1 (G2) or 4 (G4) weeks after the pig serum injection. ARB (CA; 8 mg/kg/d) administration started 1 (G3) or 4 (G5) weeks after the pig serum injection. The fibrosis areas were significantly suppressed in the PE-treated rats (G2 and 4) and the CA-treated rats (G3 and 5) compared with the pig serum–treated control group (G1). The data represent means ± SD. Each group consisted of 10 rats. *Statistically significant difference compared with the control group ($P < .01$). Abbreviations: Cont, control rats (G1); PE, PE-treated rats; CA, CA-treated rats; 1W and 4W, the inhibitor administration started 1 or 4 weeks after the pig serum injection, respectively.

TABLE 1. Effect of RAS Inhibitor on Several Markers in Animals Treated With Pig Serum

	Hydroxyproline (μg/g)	Hyaluronic Acid (ng/mL)	P-III-P (ng/mL)	7S (ng/mL)	ALT (U/L)	T Bil (mg/mL)
Pig serum	396.4 ± 104.1	107.4 ± 27.7	31.2 ± 6.2	133.2 ± 23.4	66.2 ± 12.1	0.2 ± 0.1
+PE	74.4 ± 16.4*	30.8 ± 10.7*	7.1 ± 1.8*	32.2 ± 5.4*	56.6 ± 12.1	0.2 ± 0.1
+CA	62.2 ± 4.1*	25.6 ± 10.4*	6.6 ± 1.4*	22.3 ± 4.8*	63.5 ± 15.4	0.1 ± 0.1
PBS	28.8 ± 11.3*	3.7 ± 0.7*	ND	ND	56.3 ± 12.4	0.2 ± 0.1

NOTE. Results are expressed as means ± SD (n = 10). Abbreviation: ND, not determined.
*Statistically significant difference as compared with the pig serum–treated group.

Effect of RAS Inhibitors on TGF-β_1 Expression in the Liver. Since it has been shown that TGF-β_1 acts on the activated HSCs in an autocrine manner,[26] we examined the effect of PE and CA treatment on the TGF-β_1 expression level in the liver. We performed enzyme-linked immunosorbent assay to evaluate the activated TGF-β_1 protein expression level. The TGF-β_1 protein concentration in the whole liver was significantly lower in the liver of PE-treated (G2: 28.6 ± 6.3 ng/mg wet liver, n = 10) and CA-treated (G3: 24.2 ± 4.1) rats than in the control rats (55.4 ± 8.2) ($P < .01$, Fig. 4B). We also examined the mRNA expression level of TGF-β_1 in the whole liver. As shown in Fig. 5A, mRNA expression of TGF-β_1 almost corresponded to the protein level of TGF-β_1 (Fig. 4B). The degree of TGF-β_1 protein and mRNA expression level in the liver of each group also corresponded to the number of α-SMA–positive cells.

Effect of AT-II and ARB on TGF-β_1 mRNA Expression in the Activated HSCs In Vitro. To examine whether AT-II and ARB (CA) affect the TGF-β_1 expression in the activated HSCs, we performed real-time PCR analysis. As shown in Fig. 5B, the mean TGF-β_1 mRNA expression increased 3.3-fold by the treatment with AT-II, and this effect was almost completely abolished by CA. These results suggested that the AT-II and AT_1-R interaction plays an important role in regulating TGF-β_1 expression in activated HSCs. We also examined quiescent isolated HSCs and found that TGF-β_1 mRNA was unaltered by AT-II (data not shown).

DISCUSSION

In this study, we showed that RAS inhibitory agents, ACE-I (PE) and ARB (CA), significantly blocked hepatic fibrosis induced by pig serum. Moreover, the serum levels of these inhibitors after administration of 8 mg/kg to rats were similar to those found in clinical practice.[13,14,27,28] The inhibitors were started 1 or 4 weeks after the pig serum treatment, which models the typical clinical situation better than the administration of the inhibitors at onset of fibrogenesis induction. Furthermore, these inhibitors were effective not only in the pig serum–induced model but also in fibrosis induced by a choline-deficient amino acid diet (data not shown). In short, the data appear quite relevant to clinical practice.

It has been reported that another ACE-I, captopril, inhibited the growth of fibroblasts *in vitro*.[29] It also reduced collagen accumulation in the pig serum-induced liver fibrosis model.[30] However, the degree of inhibitory effect of captopril on hepatic hydroxyproline accumulation was almost half of that of PE and CA in the present study. The authors in that study did not examine the possible involvement of AT-II, and

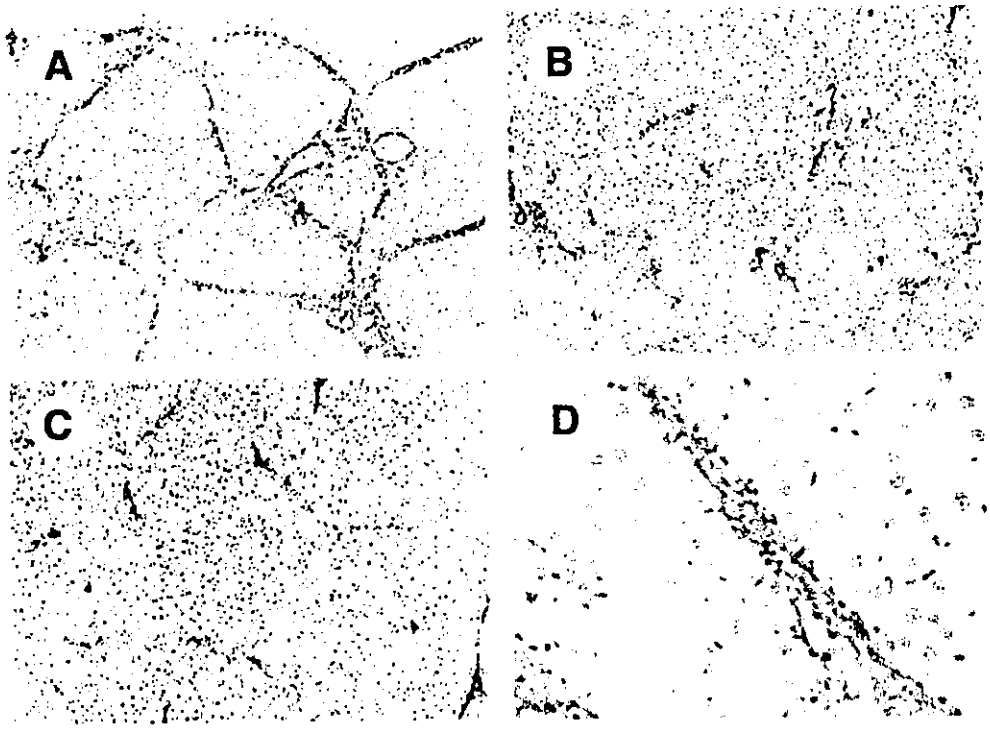

FIG. 3. Immunohistochemical analysis of α-SMA. Immunopositive cells of α-SMA were significantly suppressed in the liver of the PE-treated group (B) (G2) and CA-treated group (C) (G3) compared with the pig serum–treated control group (A) (G1). (Original magnification ×40.) (D) Localization of α-SMA–positive cells in G1. Strong immunopositive staining was observed in the fibrotic septa. (Original magnification ×200.)

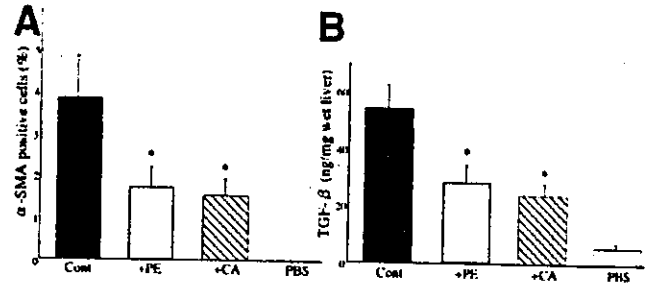

FIG. 4. (A) Computer-assisted semiquantitative analysis of α-SMA–positive cells in the liver. α-SMA–positive cells were significantly suppressed in the PE-treated group (G2) and CA-treated group (G3) compared with the pig serum–treated control group (G1). (B) TGF-β_1 protein expression levels in the liver. The TGF-β_1 protein concentration in the liver was significantly lower in the liver of the PE-treated group (G2) and CA-treated group (G3) compared with the pig serum–treated control group (G1). The suppression of TGF-β_1 with treatment of PE and CA was of similar magnitude to inhibition of α-SMA–positive cells. The data represent means ± SD. Each group consisted of 10 rats. *Statistically significant difference compared with the control group ($P < .01$). Abbreviations: Cont, control rats (G1); PE, PE-treated rats (8 mg/kg/d) (G2); CA, CA-treated rats (8 mg/kg/d) (G3).

they suggested that the attenuation by captopril was related to the reduction of mast cells and eosinophil accumulation in the hepatic tissue.

AT-II is now recognized as a multifunctional protein.[5,31] It has been reported that AT-II induced in a dose-dependent manner the vascular endothelial growth factor (VEGF), which is one of the major angiogenic factors.[32-34] The hepatic VEGF level in chronic liver diseases has been shown to increase with disease progression.[35,36] Recently, it has been reported that VEGF expression was significantly increased in the experimental liver fibrosis model.[37] VEGF stimulated HSC activation and sinusoidal capillarization.[38,39] Furthermore, it has recently been shown that anti-angiogenic compounds prevent the development of experimental liver fibrosis and HSC activation.[40] We previously reported that PE exerted an anti-angiogenic activity and that it suppressed VEGF expression.[41] It is also possible that AT-II inhibition by PE and CA

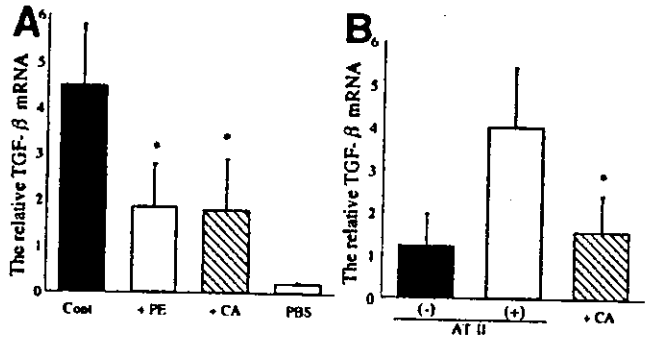

FIG. 5. The TGF-β_1 mRNA expression in the *in vivo* (A) and *in vitro* (B) experiments. TGF-β_1 mRNA expression was examined by real-time PCR as described in Materials and Methods. (A) TGF-β_1 mRNA expression in the whole liver. Abbreviations: Cont, control rats (G1); PE, PE-treated rats (8 mg/kg/d) (G2); CA, CA-treated rats (8 mg/kg/d) (G3); PBS, PBS-injected rats (G6). (B) Effect of AT-II and ARB on TGF-β_1 mRNA expression in the activated HSCs. Abbreviations: AT-II (−), untreated control group; AT-II (+), AT-II (1 μmol/L)-treated group; +CA, AT-II and CA (1 μmol/L)-treated group. The data represent means ± SD. *Statistically significant difference compared with the control group for the *in vivo* experiment and with the AT-II (+) group for the *in vitro* experiment, respectively ($P < .01$).

leads to angiogenesis and VEGF suppression and may contribute to the suppression of liver fibrosis. The interactions between these molecules are currently being analyzed in our laboratory.

In contrast to our study, some *in vitro* reports suggested that AT_1-R was not expressed in the rat HSCs, whereas it was expressed in the human-activated HSCs.[1,9,42] We could not find an exact explanation for the discrepancy. As suggested in the previous reports, however, the differences among species may have resulted from the various methodologies used by these investigators. AT_1-R in the human HSC has been proven by several methods, including receptor binding assay and changes of intracellular calcium concentration, whereas rat HSCs have been evaluated only by measuring the changes in the area of collagen lattice-containing cells.[1,9,42] The methodology for the rat HSC examination in the previous studies probably was not sensitive enough to detect the presence of AT_1-R.

It has been recently reported that TGF-β_1 is stored but not necessarily synthesized in substantial amounts in hepatocytes.[43] In this study, we did not analyze TGF-β_1 expression in the fractionated specific cell types. We found that TGF-β_1 protein and RNA changed in parallel, suggesting that HSCs were the main source of TGF-β_1 production. However, further studies, such as binding assay of rat HSCs, and the exact distribution of hepatocyte TGF-β_1 storage and HSC synthesis, have to be examined in future experiments.

In summary, we found that the AT-II and AT_1-R interaction played a pivotal role in liver fibrosis development. At clinically realistic doses, ACE-I (PE), and ARB (CA) significantly reduced liver fibrogenesis through suppressing activated HSCs with concomitant TGF-β_1 down-regulation. Because both PE and CA are widely used in clinical practice without serious side effects, these drugs may provide an effective new strategy for antifibrosis therapy.

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Endothelin-1 plays a major role in portal hypertension of biliary cirrhotic rats through endothelin receptor subtype B together with subtype A in vivo

Hideyuki Kojima*, Shinya Sakurai, Shigeki Kuriyama, Hitoshi Yoshiji, Hiroo Imazu, Masahito Uemura, Yoshihiro Nakatani, Jyunichi Yamao, Hiroshi Fukui

Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara-shi, Nara 634-8522, Japan

***Background/Aims*: Endothelin-1 has been suggested to play a key role in cirrhotic portal hypertension, but a role of its receptors in vivo is not fully elucidated.**

***Methods*: Biliary cirrhosis was induced by bile duct ligation. Expressions of endothelin-1 and its receptors were evaluated by radioimmunoassay and/or reverse-transcription polymerase chain reaction. Hemodynamics were studied using endothelin receptor agonist or antagonist.**

***Results*: Portal pressure and hepatic endothelin-1 concentrations progressively increased in parallel after bile duct ligation. Gene expression of hepatic prepro-endothelin-1 and endothelin B receptor enhanced after bile duct ligation, while that of endothelin A receptor was unchanged. Intraportal administration of endothelin-1 or endothelin B receptor agonist sarafotoxin 6c (0.5 nmol/kg, respectively) progressively raised portal pressure in both sham and cirrhotic rats. Portal hypertensive effect of sarafotoxin 6c was more intense in cirrhotic rats than sham animals. Neither endothelin A receptor antagonist FR139317 (1 mg/kg) nor endothelin B receptor antagonist BQ788 (1 mg/kg) alone ameliorated cirrhotic portal hypertension. Only the combined endothelin A and B blockade was associated with a decrease in portal pressure in cirrhotic rats.**

***Conclusions*: These results indicate that endothelin-1 plays a major role in cirrhotic portal hypertension through endothelin receptor subtype B together with subtype A in vivo.**

***Keywords*: Endothelin; Endothelin receptor; Portal hypertension; Liver cirrhosis; Bile duct ligation; RNA expression; Hemodynamic study**

1. Introduction

It is now widely recognized that cirrhotic portal hypertension is initially caused by an increased intrahepatic vascular resistance and thereafter maintained by an increased portal blood flow due to hyperdynamic circulation [1,2]. Although an increased intrahepatic vascular resistance in cirrhotic portal hypertension mainly has been attributed to the structural and irreversible changes such as fibrosis and regenerative nodules, there is a significant reversible component caused by an increased vascular tone [3,4].

Endothelin-1 (ET-1) is much more powerful vasoconstrictor than other vasoactive substances in the liver [5,6] and is of particular interest with respect to intrahepatic modulation of portal pressure [7–10]. The previous reports have shown that plasma and hepatic ET-1 concentrations are elevated in human and experimental cirrhosis [11–13] and that an exogenous ET-1 increases portal pressure in vivo as well as in an isolated perfused liver [14–17]. Recently we and other investigators reported that a mixed ET receptor antagonist ameliorated the cirrhotic portal hypertension [18–20]. These data indicate that ET-1 may play a major role in cirrhotic portal hypertension. However, a role of ET receptors in vivo mediating a vasoconstrictive property in cirrhotic liver, thus contributing to an elevated intrahepatic vascular resistance and portal hypertension is not fully clarified.

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* Corresponding author. Tel.: +81-744-22-3051; fax: +81-744-24-7122.
E-mail address: kojima@nmu-gw.naramed-u.ac.jp (H. Kojima).

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ET receptors are divided into at least two subtypes [21,22], termed type A (ET_A receptor) and type B (ET_B receptor). ET_A receptor is widely distributed on vascular smooth muscle cells and mediates the vasoconstrictive effect [21]. In contrast, ET_B receptor has been shown to locate on vascular endothelial cells and cause vasodilatation through the enhanced production of nitric oxide and/or prostacyclin [23,24]. However, recent data suggest that ET_B receptor may also exist on vascular smooth muscle cells and cause vasoconstrictive effects in some vessels [25,26]. Although it was recently shown that both ET_A and ET_B receptors mediate vasoconstriction in an isolated perfused normal liver [27], a role of ET receptors in vivo in cirrhotic portal hypertension remains to be clarified. Moreover, the expression of ET receptors in the injured liver is controversial. Leivas et al. [28] showed that RNA expression of both ET_A and ET_B receptors enhanced in human cirrhotic liver. On the contrary, another group reported that hepatic stellate cells, which modulate the sinusoidal blood flow as liver-specific pericytes, overexpressed ET-1 but not ET receptors in carbon tetrachloride treated rats [29]. A further group showed that an increase of ET receptors in hepatic stellate cells isolated from experimental cirrhotic liver is much greater for ET_B subtype than ET_A subtype [17]. These unclarified and controversial problems prompt us to determine the respective contribution of ET_A and ET_B receptors in vivo in cirrhotic portal hypertension.

2. Materials and methods

2.1. Chemicals

ET-1 and sarafotoxin-6c (S6c) were purchased from Peptide Institute (Osaka, Japan). FR139317 was kindly supplied by Fujisawa Pharmaceuticals (Tsukuba, Japan). BQ788 was purchased from Peptide International Inc. (KY).

2.2. Animal preparation

The investigation was performed in male Sprague–Dawley rats housed in a controlled environment and allowed free access to food and water until the time of study. Biliary cirrhosis with an intrahepatic portal hypertension was induced by bile duct ligation [18]. In sham rats, the bile duct was exposed but not resected. All animals received humane care and all experiments in this study were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in Nara Medical University.

2.3. Plasma and hepatic ET-1 concentrations

Plasma and hepatic ET-1 concentrations were measured in 30 rats, consisting of six sham rats, six non-operated normal rats and 18 rats with bile duct ligation (2, 3 or 4 weeks; $n = 6$, respectively). Trunk blood samples were collected in chilled tubes containing EDTA-2Na (1 mg/ml) and aprotinin (500 kIU/ml). Obtained plasma was frozen until analyzed. Liver was resected and homogenized for 1 min in 10 volumes of 1 M acetic acid and immediately heated at 100°C for 10 min. The homogenates were centrifuged at 15 000 × g for 10 min at 4°C and the resultant supernatants were stored at −20°C. ET-1 concentration was measured by radioimmunoassay [18].

Protein concentrations were determined by the method of Bradford [30] using bovine serum albumin as a standard.

2.4. RNA expressions of prepro-ET-1 and ET receptors

To evaluate RNA expressions of prepro-ET-1 and ET receptors in the liver, we performed semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis [31]. Total RNA was extracted from the pool of the same livers used for the measurement of hepatic ET-1 concentrations, using RNAzol B (TEL-TEST Inc., TX). Chloroform extraction, isopropanol precipitation and 75% ethanol washing were subsequently performed according to the manufacturer's instructions. Total RNA (3 μg) was primed with 0.5 μg oligo-dT_{12-18} primer and converted to cDNA by murine reverse transcriptase. PCR was performed using PCR Supermix (Life Technologies, Inc., MD) with the specific primers for prepro-ET-1, ET_A and ET_B receptors, which were designed according to the published sequences [32]. The sequences of the primers were as follows. Prepro-ET-1: 5′-TGCTCCTGCTCCTCCTTGAT-3′ (sense 341–360), 5′- CACCACGGGGCTCTGTAGTC-3′ (antisense 811–792); ET_A receptor: 5′-GAAGTCGTCCGTGGGCATCA-3′ (sense 538–557), 5′-CTGTGCTGCTCGCCCTTGTA-3′ (antisense 753–734); ET_B receptor: 5′-TTACAAGACAGCCAAAGACT-3′ (sense 1004–1023), 5′-CACGATGAGGACAATGAGAT-3′ (antisense 1568–1549). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. In the preliminary experiments, the proper number of amplification cycles for each gene was examined by performing RT–PCR for 15, 20, 25, 30, 35 and 40 cycles. PCR products were within the steep ascending phase of the amplification curve until 30 cycles for ET_A receptor, ET_B receptor and prepro-ET-1 and 25 cycles for GAPDH (data not shown). Prepro-ET-1 was amplified by 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 20 s and elongation at 72°C for 1 min. ET_A and ET_B receptors were amplified by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min.

2.5. Hemodynamic studies

Four weeks after the surgery, sham and biliary cirrhotic rats were anesthetized with ketamine (100 mg/kg, i.m.). PE-50 catheters were inserted into the femoral artery and vein for arterial pressure measurement and drug infusion, respectively. Portal vein was cannulated via the ileocolic vein with a double lumen catheter (outer diameter 1.0 mm) or PE-50 catheter. The femoral artery and portal vein catheters were connected to pressure transducers for blood pressure monitoring. Blood flow of superior mesenteric artery (Qsma) was measured with a flow probe (1RB; Transonic Systems Inc., NY) placed around the superior mesenteric artery using an ultrasonic transit-time technique [33].

Hemodynamic studies were performed as follows.

(1) Sham and cirrhotic rats received an intraportal administration of ET-1 or selective ET_B receptor agonist S6c (both at 0.5 nmol/kg for 1 min) through a double lumen catheter, allowing simultaneous pressure monitoring and drug infusion. Mean arterial pressure, portal pressure and Qsma were continuously monitored.

(2) Another sham and cirrhotic rats received an intravenous administration of ET_A receptor antagonist FR139317 and/or ET_B receptor antagonist BQ788 (both at 1 mg/kg) through the femoral vein catheter 5 min before hemodynamic studies. Doses of FR139317 and BQ788 were chosen according to the previous studies [34,35]. Mean arterial pressure, portal pressure and Qsma were continuously monitored.

2.6. Statistics

Results are expressed as mean ± SEM. Comparisons between groups were performed using unpaired Student's t-test. Simple regression analysis was used to evaluate the correlation between portal pressure and hepatic ET-1 concentrations. Results were considered statistically significant at $P < 0.05$.

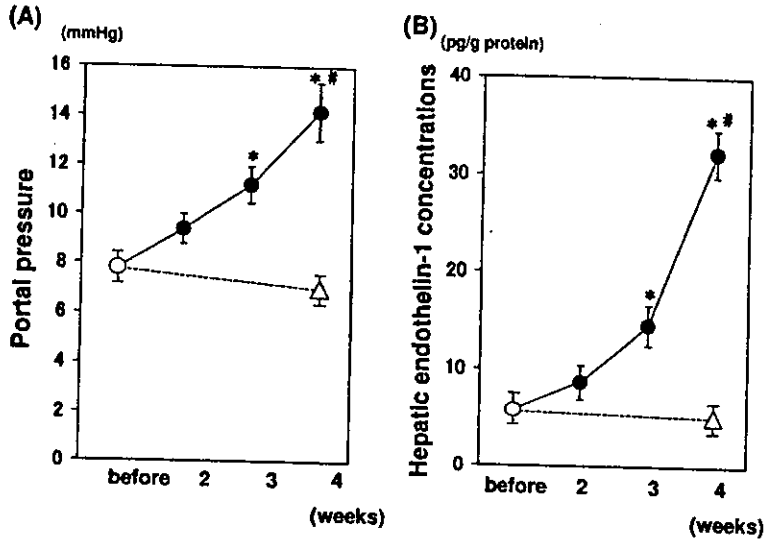

Fig. 1. Temporal change of portal pressure (A) and hepatic ET-1 concentrations (B) during the development of biliary cirrhosis. Portal pressure and hepatic ET-1 concentrations were measured in 30 rats, consisting of six sham rats (△), six non-operated rats (○) and 18 rats with bile duct ligation (●) 2, 3 or 4 weeks ($n = 6$, respectively). Values are presented as mean ± SEM. $^{\#}P < 0.01$ vs. sham rats, $^{*}P < 0.01$ vs. non-operated rats.

3. Results

3.1. Temporal changes of portal pressure and hepatic ET-1 concentrations after bile duct ligation

To clarify the relation between cirrhotic portal hypertension and ET-1, we investigated the temporal changes of portal pressure and hepatic ET-1 concentrations after bile duct ligation (Fig. 1). Portal pressure and hepatic ET-1 concentrations progressively raised in parallel after bile duct ligation. (non-operated rats, 7.8 ± 0.5 mmHg and 5.8 ± 0.3 pg/g protein; sham rats, 6.8 ± 0.4 mmHg and 4.8 ± 0.5 pg/g protein; after bile duct ligation: 2 weeks, 9.4 ± 0.6 mmHg and 8.6 ± 1.8 pg/g protein; 3 weeks, 11.2 ± 0.7 mmHg and 14.5 ± 2.1 pg/g protein; 4 weeks, 14.2 ± 1.2 mmHg and 32.2 ± 2.4 pg/g protein, respectively). There was a positive correlation ($r = 0.70$, $P < 0.01$) between portal pressure and hepatic ET-1 concentrations in the rats with bile duct ligation (Fig. 2).

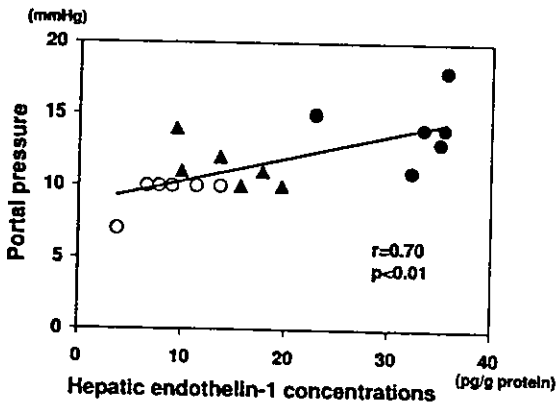

Fig. 2. The relation between hepatic ET-1 concentrations and portal pressure in the rats with bile duct ligation (○, 2 weeks; ▲, 3 weeks; ●, 4 weeks).

3.2. RNA expressions of ET-1 and its receptors

Temporal change in RNA expression of hepatic ET-1 was also investigated (Fig. 3). RT–PCR analysis of prepro-ET-1 showed a clear band with a predicted size of 471 bp. RNA expression of prepro-ET-1 in the liver enhanced in a time-dependent manner after bile duct ligation, but did not in sham rats. In addition to prepro-ET-1, RT–PCR analysis of ET_A and ET_B receptors also showed a clear single band with a predicted size of 216 and 565 bp, respectively (Fig. 4). RNA expression of ET_B receptor in the liver enhanced in biliary cirrhotic rats whose bile ducts were ligated 4 weeks ago, whereas that of ET_A receptor was unchanged.

3.3. Hemodynamic effect of ET receptor agonists in sham and cirrhotic rats

To evaluate a role of hepatic ET receptors in cirrhotic portal hypertension, we investigated the hemodynamic effect of intraportal administration of ET-1 (mixed ET_A and ET_B receptor agonist) or S6c (selective ET_B receptor agonist) in sham and biliary cirrhotic rats. Intraportal administration of ET-1 or S6c caused an initial transient decrease in systemic arterial pressure in sham and cirrhotic rats, although the magnitude of its depressor effect was much higher in S6c than ET-1 (Fig. 5A,B). In contrast, portal pressure progressively increased without an initial transient decrease, which was observed in systemic arterial pressure (Fig. 5C,D). Portal pressure reached a peak value 3–5 min after ET-1 or S6c infusion and remained to increase for 30 min in all groups. The magnitude of portal hypertensive effect of S6c was higher in cirrhotic rats than sham rats (maximum change of

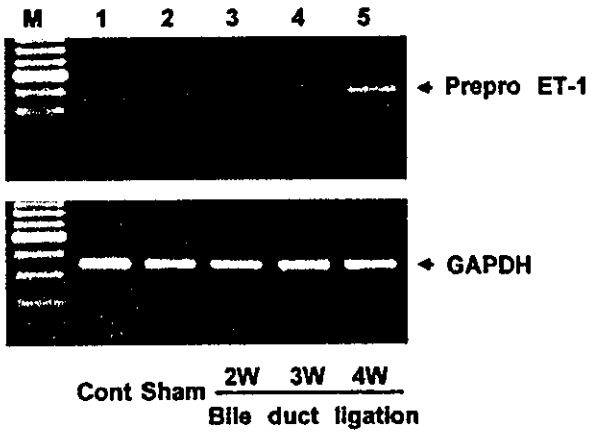

Fig. 3. RNA expression of prepro-ET-1 in the liver during the development of biliary cirrhosis. Total RNA was extracted from the pool of the same livers used for the measurement of hepatic ET-1 concentrations. RNA expression was analyzed by RT–PCR. Upper panel: prepro-ET-1; lower panel: glyceraldehyde-3-phosphate dehydrogenase as an internal control. Lane 1, non-operated rats; lane 2, sham rats; lanes 3–5, the rats with bile duct ligation 2, 3 and 4 weeks, respectively.

portal pressure, 4.6 ± 0.5 mmHg in sham rats vs. 6.6 ± 0.7 mmHg in cirrhotic rats, $P < 0.05$), whereas that of ET-1 was the reverse (maximum change of portal pressure, 5.7 ± 0.4 mmHg in sham rats vs. 3.9 ± 0.6 mmHg in cirrhotic rats, $P < 0.05$). Qsma immediately decreased after ET-1 or S6c infusion and continued to decrease for 30 min in all groups. Despite the different response in portal pressure, the response of Qsma to ET-1 or S6c did not differ between sham and cirrhotic rats (Fig. 5E,F).

3.4. Hemodynamic effects of ET receptor antagonists in sham and cirrhotic rats

To further evaluate a role of hepatic ET receptors in cirrhotic portal hypertension, we investigated the hemodynamic effect of an intravenous administration of ET_A and/or ET_B receptor antagonist in sham and cirrhotic rats. Fig. 6

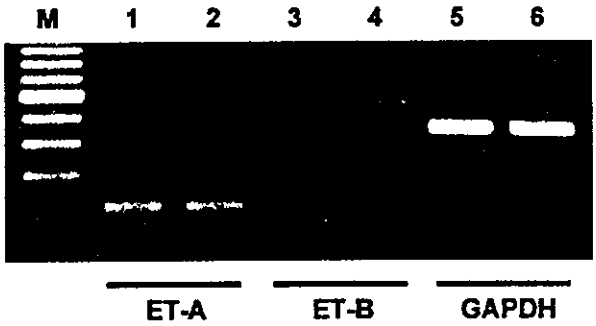

Fig. 4. RNA expression of hepatic prepro-ET-1 and ET receptors in sham and biliary cirrhotic rats with bile duct ligation 4 weeks. Lanes 1,2, ET_A receptor in sham and biliary cirrhotic rats; lanes 3,4, ET_B receptor in sham and biliary cirrhotic rats; lanes 5,6, glyceraldehyde-3-phosphate dehydrogenase as an internal control in sham and biliary cirrhotic rats.

shows a peak percent change from basal value in mean arterial pressure, Qsma and portal pressure after an infusion of FR139317 (ET_A receptor antagonist) and/or BQ788 (ET_B receptor antagonist). FR139317 did not lower portal pressure in spite of a significant reduction in Qsma in sham and cirrhotic rats (Fig. 6A). BQ788 preferentially reduced Qsma in cirrhotic rats, but influenced neither portal pressure nor systemic arterial pressure in both sham and cirrhotic rats (Fig. 6B). The simultaneous administration of FR139317 and BQ788 preferentially reduced portal pressure in cirrhotic rats, but changed neither systemic arterial pressure nor Qsma in both groups (Fig. 6C). Vehicle influenced neither mean arterial pressure, Qsma nor portal pressure in sham and cirrhotic rats (Fig. 6D).

4. Discussion

Recently the mechanism augmenting an intrahepatic vascular resistance and thus contributing to cirrhotic portal hypertension has received an increasing attention. In this respect, ET-1 is of particular interest. The liver is one of the main sites of synthesis, metabolism and action of ET-1 and this peptide is one of most powerful vasoconstrictors in the liver [5,6]. The previous reports showed that plasma and hepatic ET-1 concentrations were elevated in human and experimental liver cirrhosis [11–13] and that an exogenous ET-1 increased portal pressure in vivo as well as in isolated perfused liver [14–17]. Recently it has been reported that a mixed ET receptor antagonist decreases portal pressure in the experimental cirrhotic rats [18–20]. These data indicate that ET-1 may play a key role in cirrhotic portal hypertension. In this study, portal pressure and hepatic ET-1 concentrations progressively increased in parallel during the development of biliary cirrhosis. Moreover, portal pressure positively correlated with hepatic ET-1 concentrations. Taking into account that hepatic ET-1 is derived from sinusoidal endothelial cells and stellate cells and modulates an intrahepatic vascular resistance in a paracrine or autocrine manner [29,36], ET-1 overproduced in the injured liver may increase portal pressure along with the development of liver injury. These findings provide the further support to a major role of ET-1 in cirrhotic portal hypertension.

Although an overproduction of ET-1 in cirrhotic liver is evident, the expression of ET receptors in the injured liver are controversial. Leivas et al. [28] showed that both ET_A and ET_B receptors were overexpressed in human cirrhotic liver. Another group reported that stellate cells isolated from the experimental cirrhotic liver overexpressed ET-1, but neither ET_A nor ET_B receptor [29]. Another group showed that the magnitude of the increased ET receptor in stellate cells from the cirrhotic liver is much greater for ET_B subtype than ET_A subtype [17]. The reason for these controversial data is unclear at present. However, it is known that the expression of ET receptors on stellate cells shifts from ET_A subtype dominant to ET_B subtype dominant during

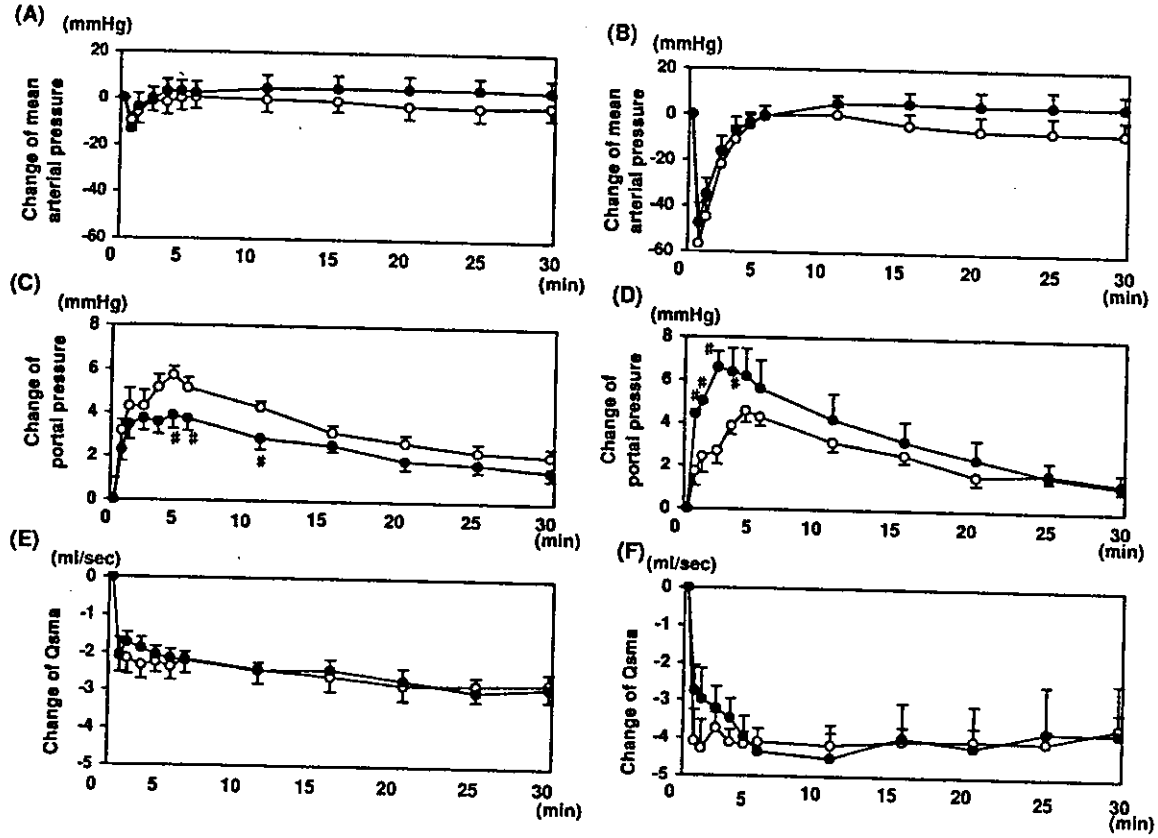

Fig. 5. Effect of ET-1 (A,C,E) and sarafotoxin-6c (B,D,F) on mean arterial pressure (A,B), portal pressure (C,D) and blood flow of superior mesenteric artery (Qsma) (E,F) in sham rats (○) and biliary cirrhotic rats (●). ET-1 and sarafotoxin-6c (each 0.5 nmol/kg body weight for 1 min) were administered through the ileocolic vein. Values are presented as mean ± SEM from eight rats. $^{\#}P < 0.05$ vs. sham rats.

cell culture [37]. This may be one reason why these controversial data may be attributed to the change of phenotype in stellate cells. In this study, we investigated the gene expression of ET receptors in a whole liver. RNA expression of ET receptors in the cirrhotic liver preferentially enhanced in ET_B receptor with the overexpression of prepro-ET-1, suggesting that an activation of this receptor subtype could be involved in the development of portal hypertension. The mechanism resulting in the overexpression of ET_B receptor in the cirrhotic liver is unclear. However, it is known that a chronic increase in blood flow induces an overexpression of ET_B receptor on hyperemic vessels and enhances the contractile effect of ET-1 in vivo [26,38]. Therefore, the increased portal blood flow, which is characteristic of cirrhotic portal hypertension, may cause the overexpression of ET_B receptor in the cirrhotic liver.

The role of ET receptors in vivo mediating a vasoconstrictive property of ET-1 in cirrhotic liver, thus contributing to the cirrhotic portal hypertension, is not fully clarified. In this study, exogenous ET-1 (mixed ET_A and ET_B receptor agonist) and S6c (selective ET_B receptor agonist) progressively increased portal pressure without an initial transient reduction, which was observed in systemic arterial pressure. This initial reduction in systemic arterial pressure has been attributed to the vasodilatation through a production of nitric oxide via ET_B receptor on vascular endothelial cells [24,35]. Thus, the absence of an initial reduction in portal pressure suggests that the net dilator effect of ET-1 and S6c is absent or weak on the portal circulation and that ET_B receptors in the portal venous system mainly mediate vasoconstriction. Moreover, two ET receptor agonists increased portal pressure in both sham and cirrhotic rats despite its depressive effect on Qsma, an important component of portal venous inflow. This finding indicates that ET receptor agonists may cause a stronger vasoconstriction in portal circulation than the splanchnic arteries, resulting in an increase of portal pressure. Interestingly, the magnitude of portal hypertensive effect by S6c was higher in cirrhotic rats than in sham rats, whereas that of ET-1 was the reverse. This result, together with the overexpression of ET_B receptor in the cirrhotic liver, suggests that an activation of this receptor subtype could be involved in the development of cirrhotic portal hypertension.

To further evaluate the role of ET receptors in cirrhotic portal hypertension, we analyzed the hemodynamic effects of ET receptor antagonists. Neither ET_A nor ET_B receptor antagonist alone ameliorated cirrhotic portal hypertension. Only the combined ET_A and ET_B blockade reduced portal pressure by approximately 20% in cirrhotic rats. This value is consistent with the effect of mixed ET receptor antagonists reported in previous studies. These findings indicate that both

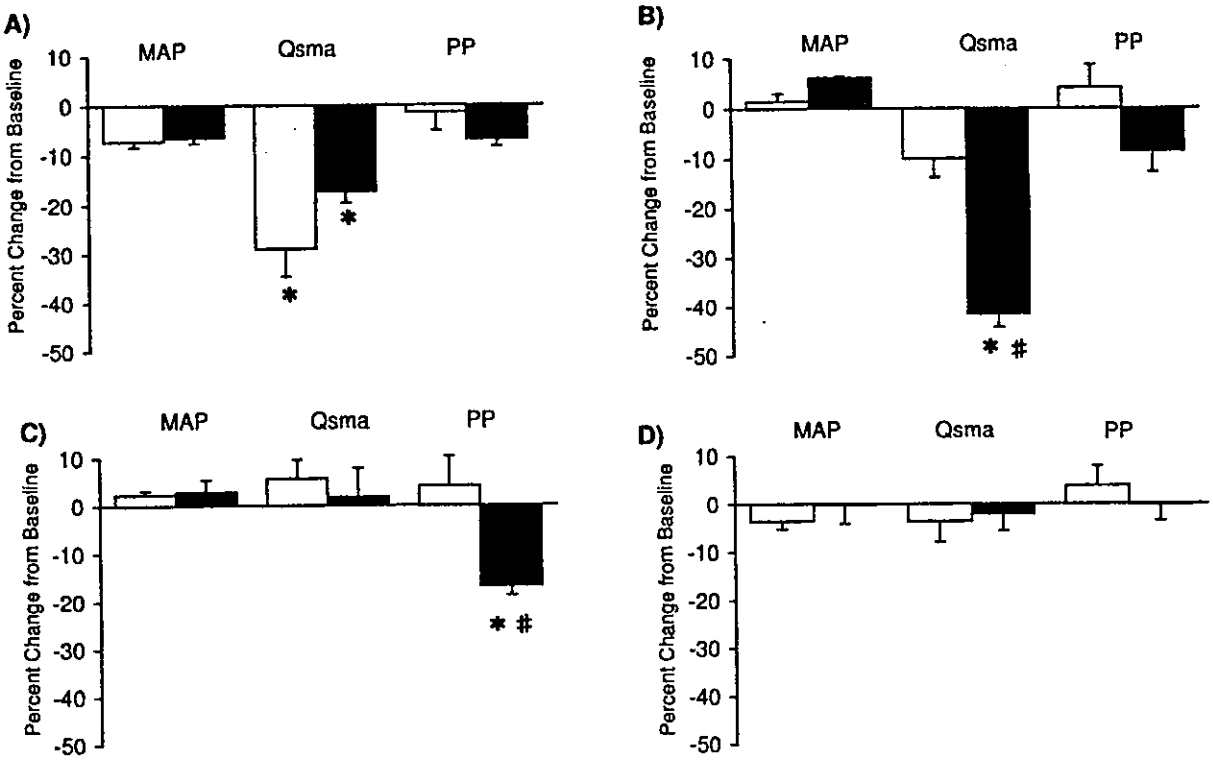

Fig. 6. Effect of ET_A receptor antagonist FR139317 and/or ET_B receptor antagonist BQ788 (A, FR139317; B, BQ788; C, FR139317 and BQ788; D, vehicle) on mean arterial pressure (MAP), blood flow of superior mesenteric artery (Qsma) and portal pressure (PP) in sham rats (□) and cirrhotic rats with bile duct ligation 4 weeks (■). FR139317 and/or BQ788 (each 1 mg/kg) was administered through the femoral vein catheter as a bolus. Values were presented mean ± SEM from eight rats. $^{\#}P < 0.05$ vs. sham rats, $^{*}P < 0.05$ vs. vehicle.

ET_A and ET_B receptors need to be blocked in order to reduce portal pressure in cirrhotic rats, and provide further support for a potential use of mixed ET receptor antagonist in the pharmacological treatment of cirrhotic portal hypertension. In this study, gene expression of ET_B receptor enhanced in cirrhotic liver and ET_B receptor agonist showed a portal hypertensive effect. On the other hand, ET_B receptor antagonist alone caused no change of portal pressure in cirrhotic rats despite a significant reduction in Qsma. This does not favor a direct role of ET_B receptors in mediating hepatic vasoconstriction, thus contributing to cirrhotic portal hypertension. However, when ET_B receptor is selectively blocked by a specific antagonist, an endogenous ET-1 may stimulate another ET receptor, ET_A receptor, and enhance an ET_A receptor-mediated vasoconstriction [19,39]. Thus, an enhanced ET_A receptor-mediated vasoconstriction and an inhibition of ET_B receptor-mediated vasodilatation following ET_B receptor blockade may decrease Qsma and offset the portal hypotensive effect due to an inhibition of ET_B receptor-mediated vasoconstriction in the liver.

Although the sites of an intrahepatic vascular resistance in cirrhotic liver are not fully clarified, the accumulating evidence suggests a significance of stellate cells in the intrahepatic circulation [14,17,29,36,40]. Hepatic stellate cells are located in the perisinusoidal space and modulate the sinusoidal blood flow as liver-specific pericytes [7,40]. This cell type possesses both ET_A and ET_B receptors [14,15] and contracts in the response to ET-1, increasing an intrahepatic vascular resistance via sinusoidal constriction [14,40]. Moreover, stellate cells proliferate and transform into highly contractile myofibroblasts during the development of liver cirrhosis [39,41]. Therefore, the sinusoidal constriction at the site of stellate cells may contribute to an increased intrahepatic vascular resistance in cirrhotic liver. Another candidate for a site of intrahepatic vascular resistance is the pre-sinusoidal portal venule. It is known that ET-1 causes both sinusoidal and pre-sinusoidal constriction and its pre-sinusoidal effect is mediated by ET_B receptors [9,14]. Thus, ETs overproduced in cirrhotic liver may induce the sinusoidal constriction via both ET_A and ET_B receptors on stellate cells and/or the pre-sinusoidal constriction via ET_B receptors in portal venules, and contribute to an increased intrahepatic vascular resistance in cirrhotic liver.

In conclusion, the results of this study indicate that ET-1 plays a major role in cirrhotic portal hypertension through ET receptor subtype B together with subtype A in vivo. These findings provide an important implication for a potential use of a mixed ET_A and ET_B receptor antagonist in the pharmacological treatment of cirrhotic portal hypertension.

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LIVER

Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis

H Yoshiji, S Kuriyama, J Yoshii, Y Ikenaka, R Noguchi, D J Hicklin, Y Wu, K Yanase, T Namisaki, M Yamazaki, H Tsujinoue, H Imazu, T Masaki, H Fukui

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See end of article for authors' affiliations

Correspondence to: Dr H Yoshiji, Third Department of Internal Medicine, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan; yoshijih@naramed-u.ac.jp

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Background: It has been shown that expression of the potent angiogenic factor, vascular endothelial growth factor (VEGF), and its receptors, flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2), increased during the development of liver fibrosis.
Aims: To elucidate the in vivo role of interaction between VEGF and its receptors in liver fibrogenesis.
Methods: A model of CCl_4 induced hepatic fibrosis was used to assess the role of VEGFR-1 and VEGFR-2 by means of specific neutralising monoclonal antibodies (R-1mAb and R-2mAb, respectively). R-1mAb and R-2mAb were administered after two weeks of treatment with CCl_4, and indices of fibrosis were assessed at eight weeks.
Results: Hepatic VEGF mRNA expression significantly increased during the development of liver fibrosis. Both R-1mAb and R-2mAb treatments significantly attenuated the development of fibrosis associated with suppression of neovascularisation in the liver. Hepatic hydroxyproline and serum fibrosis markers were also suppressed. Furthermore, the number of α-smooth muscle actin positive cells and α1(I)-procollagen mRNA expression were significantly suppressed by R-1mAb and R-2mAb treatment. The inhibitory effect of R- 2mAb was more potent than that of R-1mAb, and combination treatment with both mAbs almost completely attenuated fibrosis development. Our in vitro study showed that VEGF treatment significantly stimulated proliferation of both activated hepatic stellate cells (HSC) and sinusoidal endothelial cells (SEC). VEGF also significantly increased α1(I)-procollagen mRNA expression in activated HSC.
Conclusions: These results suggest that the interaction of VEGF and its receptor, which reflected the combined effects of both on HSC and SEC, was a prerequisite for liver fibrosis development.

It is widely recognised that hepatic fibrosis development is associated with progression of chronic liver disease.[1] It has been shown that capillarisation and phenotypic changes of the hepatic sinusoidal endothelial cells (SEC) occur during liver fibrosis development.[2–4] The hepatic SEC are unique endothelial cells (EC) that show fenestration and lack of basement membrane. They reside both morphologically and functionally apart from the ordinary EC.[5] Sinusoidal capillarisation involves changes in SEC, including loss of fenestration and deposition of a basement membrane, as well as alterations of the cell-cell and cell-matrix interactions in hepatic sinusoids.[2–4] This so called capillarisation of SEC results either from development of neovessels or from alteration of the pre-existing sinusoids, both events possibly induced in response to angiogenic stimulation.[6]

Angiogenesis is the development of new vasculature from pre-existing blood vessels and/or circulating EC stem cells.[7 8] Emerging evidence has shown that angiogenesis plays a pivotal role in many physiological and pathological processes, such as tumour growth, arthritis, psoriasis, and diabetic retinopathy.[7 9] Although previous studies conducted to determine the molecular process associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged synergistically.[4] It was shown that neovascularisation significantly increased during the development of liver fibrosis in both human and animal experimental studies.[10–12] Furthermore, a semisynthetic analogue of fumagilin, TNP-470, which possesses antiangiogenic activity, suppressed experimental liver fibrosis development.[13] These results suggest that angiogenesis also plays an important role in the development of liver fibrosis.

Angiogenesis is regulated by the net balance between proangiogenic factors and angiogenic inhibitors. To date, many positive and negative angiogenic modulating factors have been identified. Among these, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is the most potent in the angiogenesis process.[14–16] Emerging evidence has shown that VEGF plays a pivotal role in many cases of physiological and pathological angiogenesis. VEGF is not only an angiogenic factor, it is also known as a survival factor of EC.[17–19] Regarding liver fibrosis, it has been shown recently that VEGF expression significantly increased during the course of liver fibrosis development in experimental studies, and that VEGF participated in sinusoidal capillarisation in the liver.[11 12 20] In addition to hepatocytes, activated hepatic stellate cells (HSC), which play an important role in liver fibrogenesis, have been shown to increase VEGF expression during activation.[21–24]

Abbreviations: α-SMA, α smooth muscle actin; ALT, aminotransferase aspartate; A-M, Azan-Mallory; ECM, extracellular matrix; EC, endothelial cells; HSC, hepatic stellate cells; IgG, immunoglobin G; mAb, neutralising monoclonal antibody; PCR, polymerase chain reaction; P-III-P, procollagen III-N-peptide; R-1mAb, VEGFR-1 monoclonal antibody; R-2mAb, VEGFR-2 monoclonal antibody; SEC, hepatic sinusoidal endothelial cells; VEGF, vascular endothelial growth factor; VEGFR-1, fms-like tyrosine kinase (flt-1); VEGFR-2, kinase-insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1); vWF, von Willebrand factor.

Two tyrosine kinases, *fms*-like tyrosine kinase (flt-1: VEGFR-1) and the kinase insert domain-containing receptor/murine homologue, fetal liver kinase-1 (KDR/Flk-1: VEGFR-2), both of which are type III tyrosine kinase receptors, have been identified as the main VEGF receptors. By binding with high affinity to these two receptors, VEGF can stimulate EC proliferation, migration, and differentiation, and can induce angiogenesis in vitro and in vivo.[16 23] It has been shown that these two receptors serve different biological roles in many pathological events.[24–28] It has been reported that VEGFR-2 plays a more important role both in vitro and in vivo.[15 16 29] However, recent studies have revealed that VEGFR-1 also plays certain roles in pathological angiogenesis, such as tumour growth.[30–34] It has been shown that expression of VEGFR-1 and VEGFR-2 was induced during activation of HSC in vitro, although the upregulation patterns were different under different culture conditions, such as hypoxia and CCl_4 treatment.[22–34] In experimental liver fibrogenesis, it has been reported that VEGFR-1 expression increased in the liver, and VEGFR-2 was constitutively highly expressed although its expression level was not significantly altered.[11 21] The in vivo role of the interaction between VEGF and its receptor in liver fibrosis development has not yet been elucidated.

In the present study, using the specific neutralising monoclonal antibodies of VEGFR-1 and VEGFR-2, we examined the biological role of VEGF and its receptors in the progression of liver fibrosis.

METHODS

Animals

Male BALB/c mice, aged six weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless steel mesh cages under controlled conditions (temperature 23±3°C and relative humidity 50±20%), with 10–15 air changes per hour and light illumination for 12 hours a day. The animals were allowed access to food and tap water ad libitum throughout the acclimatisation and experimental periods.

Compounds and animal treatment

Anti-VEGFR-1 and VEGFR-2 specific neutralising antibodies (R-1mAb and R-2mAb, respectively) were generated as described previously.[11 33–36] Briefly, these antibodies were produced under large scale culture conditions in serum free media. The monoclonal antibodies (mAbs) were purified from conditioned media by affinity chromatography on a Gammabind-G-Sepharose column (Pharmacia Biotech, Piscataway, New Jersey, USA). The purity of the respective receptors was >99%, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and were verified to be free of endotoxin (<1 EU/ml) using a limulus amoebocyte lysate endotoxin detection kit (Pyrogen Tplus, Bio-Whittaker, Walkersville, Maryland, USA). It has been shown that R-2mAb exerts a VEGFR-2 inhibitory effect in a dose dependent manner, and that the maximal effect is achieved at a dose of 800 μg/mouse administered twice a week.[34 37] We thus employed this dose in the current study.

Mice were divided into four groups (n=10 in each group). All experimental groups received CCl_4 (2 ml/kg/body weight dissolved in 150 μl of corn oil) twice a week to develop liver fibrosis. After two weeks of treatment with CCl_4, R-1mAb and R-2mAb (800 μg/mouse) were administered intraperitoneally to group 2 (G2) and group 3 (G3) twice a week on days different from those on which CCl_4 was injected, respectively. In group 4 (G4), both R-1mAb and R-2mAb were administered simultaneously. Animals in group 1 (G1) received the same amount of control immunogloblin G (IgG) as described previously.[35 36] Mice which received only corn oil were examined as a negative control group. After eight weeks of treatment with CCl_4, all mice were killed under anaesthesia.

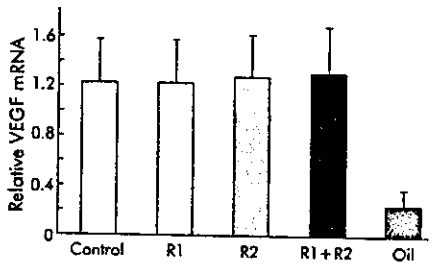

Figure 1 Vascular endothelial growth factor (VEGF) mRNA expression in the CCl_4 treated liver. VEGF mRNA expression was examined by real time polymerase chain reaction as described in the methods section. Hepatic VEGF expression increased during liver fibrosis development. Neither R-1mAb nor R-2mAb treatment altered VEGF gene expression during development of fibrosis. Control, immunogloblin G treated mice (800 μg/mouse) (G1); R1, R2, R-1mAb and R-2mAb treated mice (800 μg/mouse) (G2 and G3, respectively); R1+R-2, R-1mAb and R-2mAb combination treated group (G4); Oil, corn oil injected negative control mice. Data are means (SD) (n=5).

All animal procedures were performed according to approved protocols and in accordance with the standard recommendations for the proper care and use of laboratory animals.

Histological and immunohistochemical examinations

In all experimental groups, 5 μm thick sections of formalin fixed and paraffin embedded livers were processed routinely for Azan-Mallory (A-M) staining for determination of liver fibrosis development. Immunohistochemical staining of α smooth muscle actin (α-SMA) was performed as previously described using paraffin embedded sections with a primary anti-α- SMA antibody (Dako, Kyoto, Japan).[38–40] Semiquantitative analyses of fibrosis development and the immunoposltive cell area were carried out with the Fuji-BAS 2000 image analysing system (Fuji, Tokyo, Japan) in six ocular fields (40× magnification) per specimen from five mice. We did not count α-SMA positive vessels in the portal area which were assumed to be hepatic arteries. We only included α-SMA positive cells in the sinusoidal lining for image analysis.[38–40]

Hepatic hydroxyproline content and serum markers

Hepatic hydroxyproline content was determined as previously described with 200 mg of frozen samples.[40] The hydroxyproline content was expressed as μg/g wet liver. Alanine aminotransferase aspartate (ALT) and total bilirubin were assessed using routine laboratory methods. Serum hyaluronic acid and procollagen III-N-peptide (P-III-P) were also measured as described previously.[38]

Immunoprecipitation

To determine whether R-1mAb and R-2mAb at a dose of 800 μg/mouse suppressed autophosphorylation of the respective receptors in the liver, immunoprecipitation was performed as previously described[11] Fifteen minutes after R-1mAb and R-2mAb were injected intraperitoneally, the liver was resected from three mice in each group and snap frozen immediately. The liver pool lysate solution was concentrated and used for immunoprecipitation. To conduct immunoprecipitation, liver lysates were immunoprecipitated with antiphosphotyrosine before conducting sodium dodecyl sulphate- polyacrylamide gel electrophoresis. Antityrosine (4G10) was purchased from Upstate Biotechnology (New York, USA) and anti-VEGFR-2 (C-1158) and VEGFR-1 (C-17) were obtained from Santa-Cruz (California, USA). Before western blotting, we stained each membrane with Ponceau solution (Sigma, Michigan, USA) to confirm that the same amounts of protein were immunoprecipitated (data not shown). The blots were developed using an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad, California, USA).

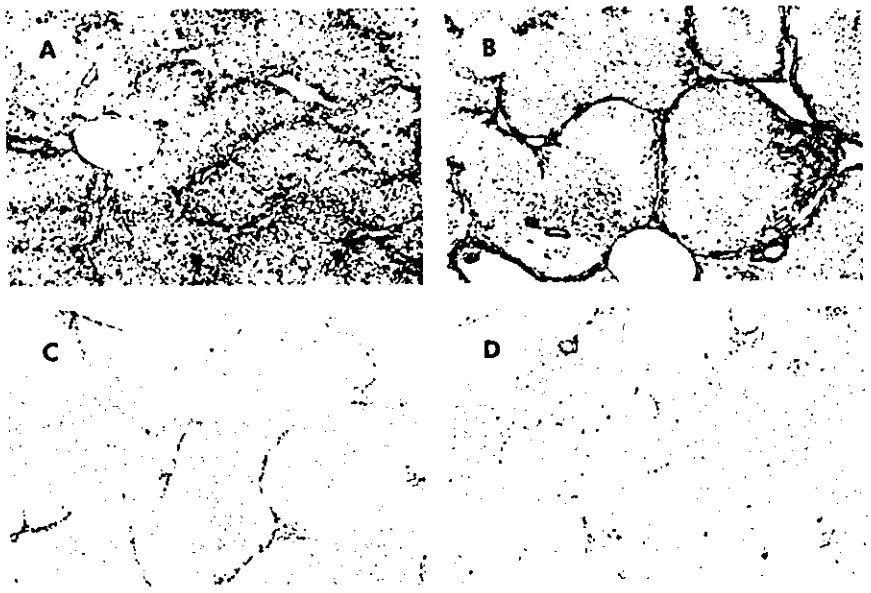

Figure 2 Microphotographs of liver sections from CCl_4 treated mice. (A) Control immunoglobin G treated group after CCl_4 treatment (800 μg/mouse) (G1). (B, C) R-1mAb and R-2mAb treated (800 μg/ mouse) groups (G2 and G3, respectively). (D) R-1mAb and R-2mAb combination treated group (G4). The livers in G1 show extensive fibrosis development. In G2 and G3, liver fibrosis development was significantly attenuated, and the inhibitory impact was more potent with R-2mAb treatment than with R-1mAb treatment. Fibrosis development was almost completely abolished in the livers of G4 (A-M staining, 40×).

RNA expression of VEGF, CD-31, and α1-(I)-procollagen by real time polymerase chain reaction

The VEGF, CD-31, which is used widely as a marker for neovascularisation, and α1-(I)-procollagen mRNA expression were evaluated by real time polymerase chain reaction (PCR), as described previously.[38 41] mRNA was extracted from the whole liver of the animals in each experimental group (n=5). For cDNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer's manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, California, USA), which was used for real time PCR amplification following the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). Relative quantitation of gene expression was performed as described in the manual, using glyceraldehyde-3-phosphate dehydrogenase as an internal control. The threshold cycle and standard curve method were used for calculating the relative amount of the target RNA, as described for PE. The following temperatures were employed: hold at 50°C for two minutes, hold at 60°C for 30 minutes, hold at 94°C for five minutes, cycle 45 repeats at 94°C for one minute, at 55°C for one minute, and at 72°C for one minute. 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.

Isolation and culture of HSC and SEC

R-1mAb and R-2mAb only react with mice receptors and not with those of other species, such as the rat.[31 42] Although we attempted several times to isolate pure HSC from the liver of mice, contamination with other types of non-parenchymal cells, such as EC, could not be ruled out. Furthermore, the yield of purified HSC from mice was too low to perform several experiments, as described previously.[38] We thus employed HSC from the liver of the rat, and examined the effect of VEGF treatment on VEGF-receptor interaction in activated HSC.

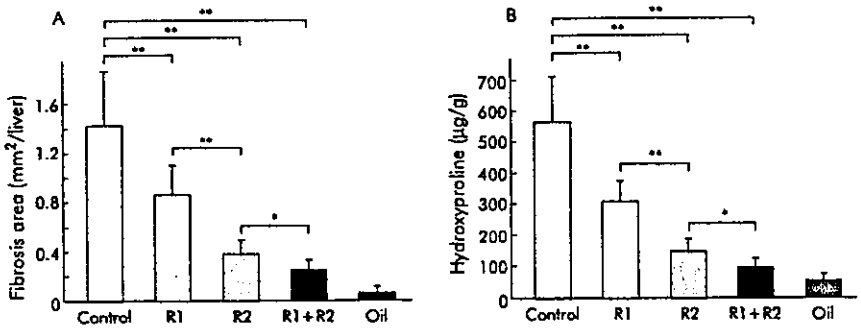

Figure 3 Effects of R-1mAb and R-2mAb on fibrosis area (A) and hepatic hydroxyproline content (B) in the CCl_4 treated liver. (A) Fibrosis area was evaluated by an image analyser, as described in the methods section. R-1mAb and R-2mAb significantly suppressed liver fibrosis development compared with the control group ($p<0.01$), and the inhibitory impact was more potent with R-2mAb treatment than that with R-1mAb treatment ($p<0.01$). The combination treatment with both mAbs revealed further inhibition compared with that of R-2mAb alone ($p<0.05$). (B) The inhibitory effects of R-1mAb and R-2mAb on hepatic hydroxyproline content exerted behaviours similar to those on fibrosis area. 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.

Table 1 Effect of R-1 and R-2 mAb on several markers in animals treated with CCl_4

Group	Hyaluronic acid (ng/ml)	P-III-P (ng/ml)	ALT (U/ml)	Total bilirubin (mg/dl)
Control	190.2 (27.5)	46.3 (6.4)	242.4 (51.8)	1.4 (0.2)
R1 mAb	82.7 (17.4)**	20.6 (6.0)**	216.4 (55.7)	1.1 (0.2)
R2 mAb	28.7 (14.3)††	7.1 (3.4)††	233.8 (60.8)	1.2 (0.2)
R1+R2	18.6 (7.9)	3.8 (1.9)	226.3 (54.5)	1.1 (0.3)
Oil	11.4 (3.0)	2.4 (1.1)	45.1 (7.3)	0.6 (0.2

Data are means (SD) (n=8).
P-III-P, procollagen III-N-peptide; ALT, aminotransferase aspartate; R1 mAb, VEGFR-1 monoclonal antibody; R2 mAb, VEGFR-2 monoclonal antibody; R1+R2, R1 mAb+R2 mAb.
**p<0.01 compared with the control group; ††p<0.01 compared with the R1 mAb treated group.

Liver HSC were isolated from the liver of F344 rats, as described previously,[38] with a minor modification. Briefly, the liver was perfused with Krebs-Ringer solution followed by 0.1% pronase E and 0.032% collagenase (Nakarai, Kyoto, Japan) solution at 37°C. The digested liver was cut, minced, and incubated in Krebs-Ringer solution containing 0.08% pronase E, 0.04% collagenase, and 20 µl/ml DNase for 30 minutes at 37°C (pH 7.3). After passage through a nylon mesh, cells were centrifuged at 450 *g* for eight minutes. The HSC enriched fraction was obtained by centrifugation in 8.2% Nycodenz (Nycomed Pharma AS, Oslo, Norway) solution at 1400 *g* for 20 minutes. HSC in the upper white layer were washed by centrifugation at 450 *g* for eight minutes and suspended in DMEM medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mU/ml streptomycin. Cell viability was over 95%, as determined by the Trypan blue exclusion test. Cells were plated at a density of 5×10^5 cells/ml on either collagen I, an established model of culture activation,[43] or basement membrane-like EHS matrix for six days in the presence of 20% fetal calf serum, serum starved for 48 hours, and treated with doses of 10 and 100 ng/ml of human recombinant VEGF (R&D systems Inc. Minneapolis, Minnesota, USA), as described previously.[34] SEC were isolated from the rat liver with collagenase followed by differential centrifugation, as described previously,[44] and grown in HuMedia-EG2 medium (Kurabo, Osaka, Japan) on a collagen I coated dish supplied with human recombinant VEGF (10 ng/ml) as VEGF is a prerequisite for SEC survival.[45]

In vitro proliferation assay

The effects of VEGF on in vitro proliferation of activated HSC and SEC were determined using the MTT assay, as described previously.[46] Briefly, cell proliferation was quantified via conversion of tetrazolium, 3-(-4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by cells cultured in 96 well plates. Absorbance with a 540 nm filter represents conversion to formazan, which is directly proportional to the number of living cells. Absorbance was read with an ELISA plate recorder (n=6 per group).

Statistical analysis

To assess the statistical significance of intergroup differences in quantitative data, the Mann-Whitney U test was used to compare mean values between the two groups. The Kruskal-Wallis test was used to compare mean values between more than two groups.

RESULTS

VEGF mRNA expression

Firstly, we examined VEGF mRNA expression during CCl_4 induced liver fibrosis development. Similar to previous reports,[11 12] hepatic VEGF expression significantly increased during liver fibrosis development after CCl_4 treatment. Neither R-1mAb nor R- 2mAb treatment altered VEGF gene expression during development of fibrosis (fig 1). We performed a preliminary routine reverse transcription PCR, and found that among the alternative splicing mouse VEGF genes, $VEGF_{164}$ and $VEGF_{120}$ were abundant in the CCl_4 treated liver (data not shown).

Histological findings and fibrosis markers

A-M staining revealed that eight weeks of treatment with CCl_4 resulted in marked liver fibrosis development (fig 2A). Both R-1mAb and R-2mAb treatment significantly suppressed fibrosis development (fig 2B, 2C, respectively), and the combination treatment of R-1mAb and R-2mAb almost completely attenuated CCl_4 induced liver fibrosis (fig 2D). No fibrosis development was found in the corn oil treated group, and the low dose of R-1mAb (400 µg/mouse) did not exert such an inhibitory effect (data not shown). Densitometric analysis showed that the fibrosis areas (fig 3A) mostly corresponded to the histological findings. Although both R-1mAb and R-2mAb significantly suppressed liver fibrosis development compared with the control IgG treated group ($p<0.01$), the inhibitory impact was more potent with R-2mAb than with R-1mAb treatment ($p<0.01$). The combination treatment with both mAbs revealed further inhibition compared with that of R-2mAb alone ($p<0.05$). Hepatic hydroxyproline content showed similar results to those of fibrosis area (fig 3B). These results suggest that both VEGFR-1 and VEGFR-2 play important roles in liver fibrogenesis, and that the role of VEGFR-2 is more predominant than that of VEGFR-1. The serum fibrosis markers, hyaluronic acid and P-III-P, were also significantly suppressed by treatment with R-1mAb and R-2mAb, whereas serum ALT and total bilirubin levels did not change with the use of R-1mAb and R-2mAb (table 1). We also examined the

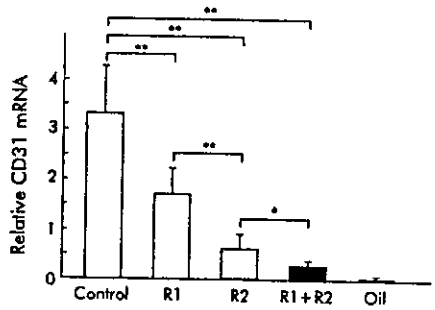

Figure 4 Effects of R-1mAb and R-2mAb on neovascularisation in the liver. CD31 mRNA expression was examined by real time polymerase chain reaction, as described in the methods section. CD31 gene expression was significantly increased during liver fibrosis development. Treatment with R-1mAb and R-2mAb significantly attenuated neovascularisation in the liver. Suppression of angiogenesis by treatment with R-1mAb and R-2mAb was of a similar magnitude to that of inhibition of fibrosis areas. Control, immunoglobulin 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.