Introduction

Hepatic stellate cells have now been clearly identified as the primary cellular source involved in the pathogenesis of liver fibrosis (Friedman, 1991). During the development of liver fibrosis, stellate cells undergo activation, a process characterized by increased cell proliferation, morphological transformation into myofibroblast-like cells and synthesis of excessive extracellular matrix components (Friedman, 1991). These cells are also able to synthesize enzymes, called matrix metalloproteinases (MMPs), which can degrade matrix proteins in the extracellular space. The activity of MMPs is carefully regulated by controlling their conversion from pro-enzymes to the catalytic form and by a family of specific inhibitors, called tissue inhibitors of metalloproteinases (TIMPs).

On the other hand, many studies of signal transduction are now underway. The MAPK family of protein kinases includes the extracellular signal-regulated kinases (ERKs) (Boulton et al., 1991) the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) (Derijard et al., 1994; Kyriakis et al., 1994), and p38 (Han et al., 1994) The MAPK signaling pathway is a multistep phosphorylation cascade that transmits signals from the cell's surface to cytosolic and nuclear targets (Cohen, 1997). It has been suggested that not only do MAPKs serve as the signaling pathways production of MMPs (Esparza et al., 1999; Zeigler et al., 1999), but that they also participate in production of TIMPs (Li and Zafarullah, 1998; Eberhardt et al., 2000). However, the role of ERK, SAPK and p38 pathways in regulating production of MMPs and TIMPs by HSCs is not well known at present.

Many agents have been proposed for the prevention and treatment of fibrosis but no specific inhibitor of stellate cell activation has yet been developed.

TJ-9 has been reported, including by us, to prevent fibrosis via the inhibition of HSCs in different animal models of fibrosis due to choline-deficiency (Sakaida et al., 1998a,b), and pig serum (Shimizu et al., 1999), as well as in isolated stellate cells (Kayano et al., 1998). TJ-9, commonly prescribed in Japan as Sho-saiko-to, is the most popular herbal medicine in Japan and has been widely used in the treatment of chronic liver diseases, especially chronic viral hepatitis. TJ-9 consists of an aqueous extract from the roots of scutellaria, glycyrrhiza, bupleurum, and ginseng; the pinella tuber; the jujube fruit; and the thew ginger rhizome. A number of studies have indicated its cytoprotective effects in experimental liver injuries (Yamamoto et al., 1985; Araki et al., 1988), cancer-preventive effects (Okita et al., 1993, 1994; Tatsuta et al., 1991; Oka et al., 1995), anti-tumor, and apoptotic effects (Matsuzaki et al., 1996; Yano et al., 1994).

From the clinical point of view, treatment for liver fibrosis has been mainly focused on prevention of stellate cell activation instead of acceleration of degradation.

To gain further insights into the effect of TJ-9 on suppression of hepatic fibrosis, we examined changes of the MMPs/TIMPs balance and MAPK pathways of TJ-9 treated HSCs.

Materials and methods

Animals

Male Wistar rats weighting 400 to 450 g (Nippon SLC Co., Ltd., Shizuoka, Japan) were obtained, quarantined for 1 week, and housed in a room under controlled temperature (25 °C), humidity, and

lighting (12 hours light, 12 hours dark). Access to food and tap water was ad libitum. After a 1-week acclimation period on a basal diet (Oriental MF Diet; Oriental Yeast Company, Japan), the rats were used for the experiments.

Isolation and culture of rat hepatic stellate cells

Rat hepatic stellate cells (HSCs) were isolated with Nycodenz as described previously (Kayano et al., 1998).

Yields were 1.0-1.5 $\times$ 10^7 cells/rat. Cell viability was always over 95% as determined by the trypan blue exclusion test. Cell purity was more than 90% as assessed by the presence of yellow droplets and desmin immunoreactivity. Then isolated hepatic stellate cells were cultured at a density of 5.0 $\times$ 10^5 cells/ml in monolayer culture on uncoated 60-mm plastic dishes (Iwaki Glass Co., Ltd., Tokyo, Japan). All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After incubation for 4 h, non-adherent cells were removed with a pipette and the culture medium was replaced with medium containing various concentrations of TJ-9 or medium alone (control). In some experiments, SB203580 or PD98059 (Calbiochem, La Jolla, CA) was added in addition to TJ-9. SB20380 and PD980597 were dissolved in DMSO at the final concentration of 0.1%. SB20238 is known as a highly specific inhibitor of p38 kinase (IC_{50} = 34nM in vitro, 600 nM in cells) and even 100 μM does not significantly inhibit JNK and p42 MAP kinase, and PD98059 is known as specific inhibitor of ERK kinase (IC_{50} = 2 μM), according to the manufacturer's instructions.

The medium was changed every 24 h and cell culture was continued up to 4 days (serum was depleted 24 h before the assessment).

The treated HSCs were scraped after being washed with PBS twice and suspended at 4 °C in 0.5 ml of PBS with 0.1% Triton X. An equal amount of total protein (100 μg) was applied for the quantitative analysis of type IV collagen-degrading activity, zymography and reverse zymography.

Preparation of culture medium with Sho-saiko-to (TJ-9)

Sho-saiko-to (TJ-9, powder) was kindly provided by Tsumura Co., (Tokyo, Japan). Water-soluble ingredients of TJ-9 were obtained as described previously (Kayano et al., 1998). The final concentrations of TJ-9 used were 10, 100, 500 and 1000 μg/ml. The PH values of all culture media with or without TJ-9 were adjusted to within the physiological range.

Hybridization probes

The following probes were used in this study. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) (Sakaida et al., 1996, 1998a,b) was purchased from American Type Culture Collection (Rockville, MD, USA). TIMP-1 cDNAs was used as described (Sakaida et al., 1999). MMP-2 cDNA was a generous gift of Dr. Hiroshi Sato (Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Ishikawa-Pref., Japan) (Sato et al., 1994). MMP-13 cDNA was the generous gift of Dr Cheryl O. Quinn (Pediatric Research Institute, Department of Pediatrics, Health Sciences Center, Saint Louis University, MO, USA) (Hironaka et al., 2000).

Northern blot analysis

Northen blot analysis was performed after the isolation of 10 μg of total RNA from isolated hepatic stellate cells as described previously (Hironaka et al., 2000). Each signal strength of mRNA was determined after normalization by relevant G3PDH mRNA levels.

Quantitative analysis of type IV collagen-degrading activity

Type IV collagen-degrading activity in conditioned media was determined as described previously (Hironaka et al., 2000) using a Type IV collagenase activity assay kit (YU-18001, Yagai Co., Kamagata, Japan). The principle of the type IV collagenase activity assay kit is based on fluorescent measurement of collagen fragments upon cleavage by gelatinases. It is known that collagen decomposition fragments differ from whole collagen in their temperature of denaturation and ethanol solubility. Upon gelatinase cleavage of fluorescently-labeled collagen type IV, decomposition fragments are produced. These fragments are selectively denatured and extracted with ethanol. The fluorescence intensity of the extracted product is measured and correlates with type IV collagen-degrading activity.

Zymography

As described by Durko et al. (Durko et al., 1997), SDS-polyacrylamide (10%) gels were co-polymerized with gelatin at a final concentration of 1 mg/ml. The concentrated conditioned media were mixed 3:1 (v/v) with sample buffer (0.3 M Tris-HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue and 40% glycerol), loaded onto the gels without boiling and separated by electrophoresis. The gels were washed for 1 h in a solution of 2.5% Triton X-100 in 40 mM Tris-HCl, pH 7.6, and for 15 min in 10 mM Tris-HCl, pH 8. For the enzymatic reaction to take place, the gels were incubated for 18 h at 37 °C in a solution of 50 mM Tris-HCl, pH 8, containing 10 mM $CaCl_2$. The gels were stained for 2 h in a 0.5% Coomassie blue R250 solution, then destained in 20% methanol with 10% acetic acid until clear bands (indicating lysis) were apparent on the blue background. Prestained molecular weight markers were resolved on the same gels, separated from other samples after electrophoresis and fixed in 5% acetic acid. To characterize the protease bands, some of the gels were incubated in a buffer (50 mM Tris-HCl, pH 8, with 10 mM CaCl2) containing 20 mM EDTA (a metalloproteinase inhibitor).

Reverse zymography

Reverse zymography was performed as previously described (Herron et al., 1986). Gelatinase inhibitory activity was detected by incubating standard gelatin zymograms for 1 h at 37 °C in a preparation of purified collagenase (2 unit/ml) before incubation at 37 °C for 18 h in a solution of 50 mM Tris-HCl, pH 8, containing 10 mM $CaCl_2$. Gels were stained and destained as described above, after which stained bands manifested the presence of gelatinase inhibitory activity corresponding to TIMPs in the polyacrylamide gels.

Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using Student's t-test and one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered to be statistically significant.

Ethical considerations

This experiment was reviewed by the Committee of Animal Experiment Ethics in Yamaguchi University School of Medicine and was carried out under the Guidelines for Animal Experiments in Yamaguchi University School of Medicine (No. 105) and Notification (No. 6) of the Japanese Government.

Results

Effects of TJ-9 on MMP-2, 13 and TIMP-1 mRNA expression of activated HSC

Messenger RNA expression was investigated by Northern blot analysis on day 4 after culture. Under control culture conditions (no treatment), HSCs expressed MMP-2, 13 and TIMP-1 mRNAs (Fig. 1A, 1B, lane 1). 500 μg/ml of TJ-9 significantly elevated MMP-2 transcripts expressed at 3.1 kb and strikingly inhibited TIMP-1 mRNA expression (Fig. 1A, 1B, line 2). Expression of MMP-13 mRNA with stellate cells was dramatically reduced in a time-dependent manner after isolation. Although MMP-13 mRNA expression was not detected after 4-day culture, addition of 500 μg/ml of TJ-9 increased MMP-13 mRNA expression to a detectable level, though it was still very weak (Fig. 1A, 1B). Reduction of type I procollagen mRNA expression could be reproduced with 500 μg/ml of TJ-9 as previously reported (Kayano et al., 1998).

Morphologically, TJ-9 seemed to prevent the transformation to myofibroblast-like cells as previously reported (data not shown) (Kayano et al., 1998).

The doses up to 1000 μg/ml of TJ-9 showed no cytotoxicity with cultured stellate cells.

Effects of TJ-9 on MMP-2 and TIMPs production by activated HSC

When serum-free conditioned media derived from HSCs were analyzed on polyacrylamide gels co-polymerized with 1 mg/ml gelatin, the band of lysis was observed in the zymograms. These gelatinolytic activities were completely inhibited when the gels were incubated in the presence of 20mM EDTA (data not shown), indicating that they were mediated by metalloproteinases. TJ-9 conditioned media exhibited gelatinase activities in a dose-dependent manner on gelatin zymograms, seen mainly as a triplet with bands at molecular masses of 70, 66 and 62kDa (Fig. 2A). The 66 kDa gelatinase predominanted, and the 62 kDa band was at the limit of detection. These bands migrated in the Mr 62-70 kDa region corresponding to the predicted molecular weights for MMP-2. The appearance of these bands was dependent on the concentration of TJ-9, and gelatinase activity reached a peak at the concentration of 500 or 1000 μg/ml. Upregulation of MMP-2 production was also confirmed by quantitative measurement using the type IV collagen-degrading assay. Collagen IV

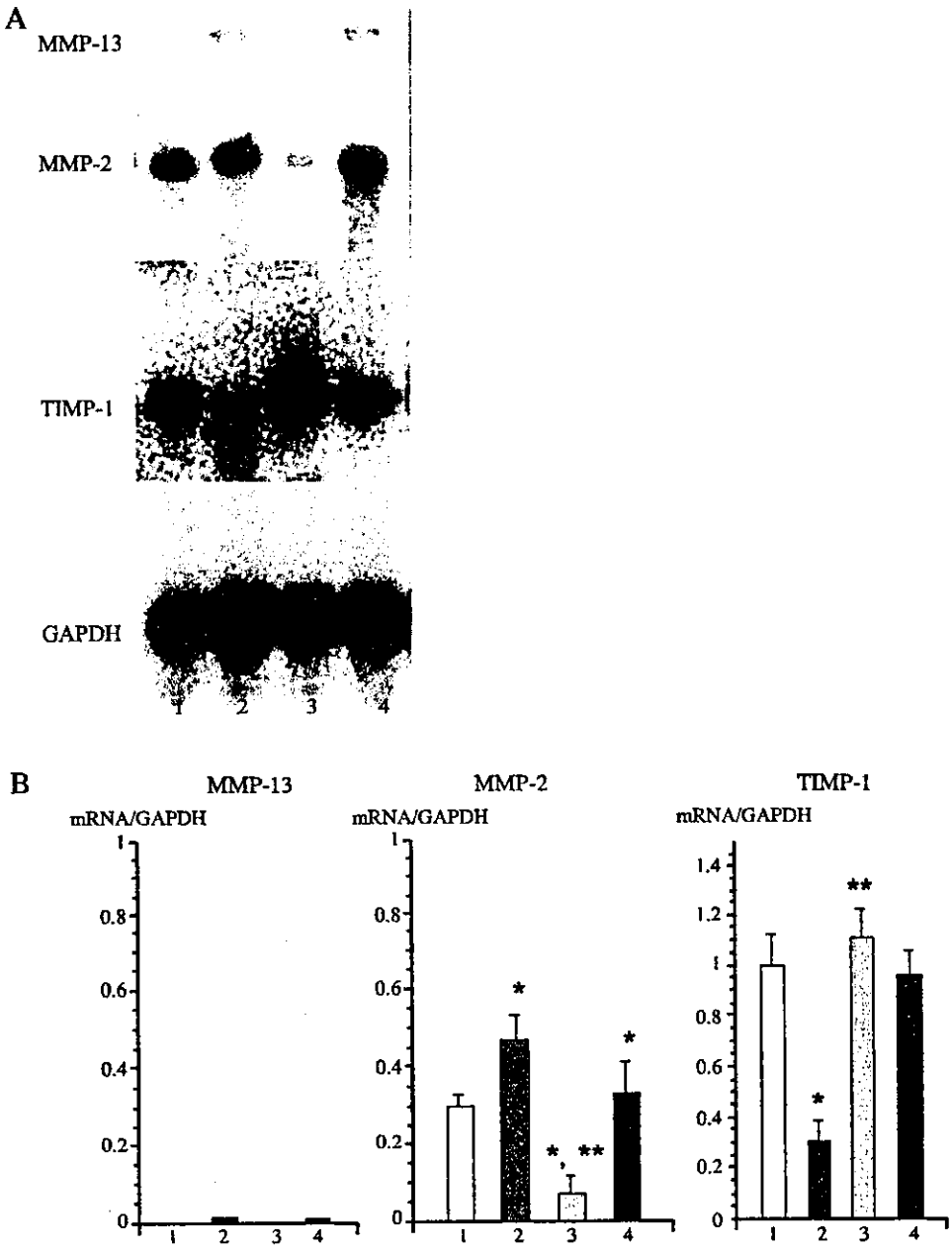

Fig. 1. A. Messenger RNA expression of MMP-13, MMP-2, TIMP-1 and G3PDH. Freshly isolated hepatic stellate cells were cultured for 4 h. The medium was replaced without TJ-9 (lane 1), with 500 μg/ml TJ-9 (lane 2), with 500 μg/ml TJ-9 plus 10 μM SB203580 (lane 3) and with 500 μg/ml TJ-9 plus 50 μM PD 98059 (lane 4). The medium was changed every 24 h. After 4 days, mRNA expression was examined. The figure shows a representative example of 5 independent Northern blots. B. Graphic representation of Fig. 1. The results of the densitometric analysis after normalization against hybridization signals for G3PDH are shown mean ± SD of 5 independent experiments. * $P<0.01$ vs lane 1 (control), ** $P<0.01$ vs lane 2 (TJ-9 500 μg/ml).

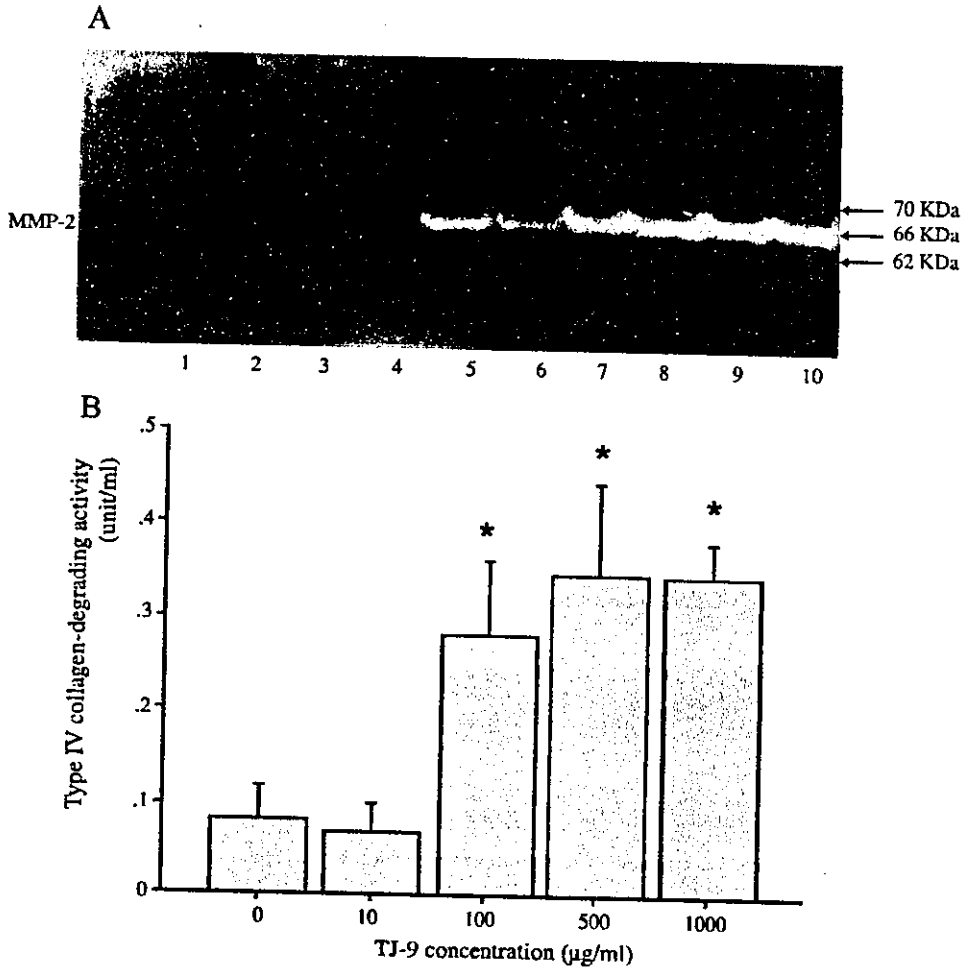

Fig. 2. A. Zymography with various concentrations of TJ-9. Freshly isolated hepatic stellate cells were cultured for 4 h. The medium was replaced without TJ-9 (lane 1, 2), or with 10 μg/ml (lanes 3, 4), 100 μg/ml (lanes 5, 6). 500 μg/ml (lanes 7, 8), and 1000 μg/ml (lanes 9, 10) of TJ-9. After 4 days, zymography was performed. B. Quantitative analysis of type IV collagen-degrading activity was done instead of zymography shown in Fig. 2-A. The results are shown as mean ± SD of 5 independent experiments. * $P<0.01$ vs 0 (control).

decomposition by serum-free conditioned media derived from HSCs increased dose-dependently and reached a peak at the concentration of 500 μg/ml of TJ-9, consistent with zymograms (Fig. 2B).

TJ-9 markedly reduced the activity of TIMPs from HSC in a dose-dependent manner as shown by a reverse zymogram (Fig. 3). This inhibitory effect on the activity of TIMP-1, 2 reached a peak at the concentration of 500 μg/ml of TJ-9. Thus, the TJ-9-enhanced gelatinolytic activity in culture media could be mainly attributed to the decreased synthesis of TIMPs in addition to the increased MMP-2 production.

Although the changes of MMP-2 or TIMP-1 mRNA expression were not remarkable, the treated cells showed prominent changes of activity, which is more physiologically relevant.

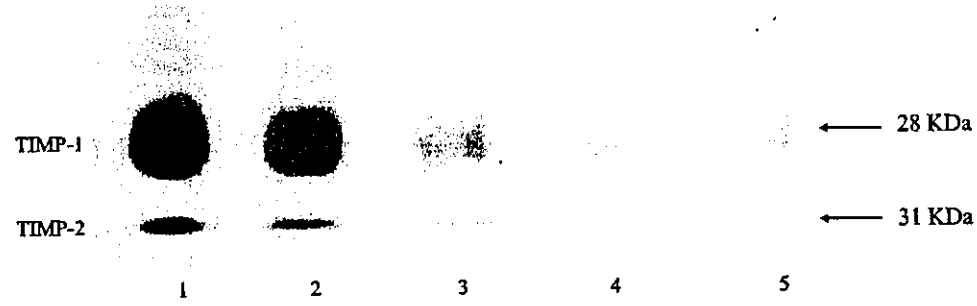

Fig. 3. Reverse zymography with concentrations of TJ-9. Freshly isolated hepatic stellate cells were cultured for 4 h. The medium was replaced without TJ-9 (lane 1), or with 10 μg/ml (lane 2), 100 μg/ml (lane 3). 500 μg/ml (lane 4), and 1000 μg/ml (lane 5) of TJ-9. After 4 days, reverse zymography was performed. The figure shows a representative example of 5 independent experiments. * $P < 0.01$ vs 0 (control).

Effect of inhibitors of MAPKs on expression of MMPs and TIMPs

The functional role of MAP kinases in mediating the down-regulatory effect of TJ-9 on expression of TIMPs and the up-regulatory effect on MMPs expression were examined.

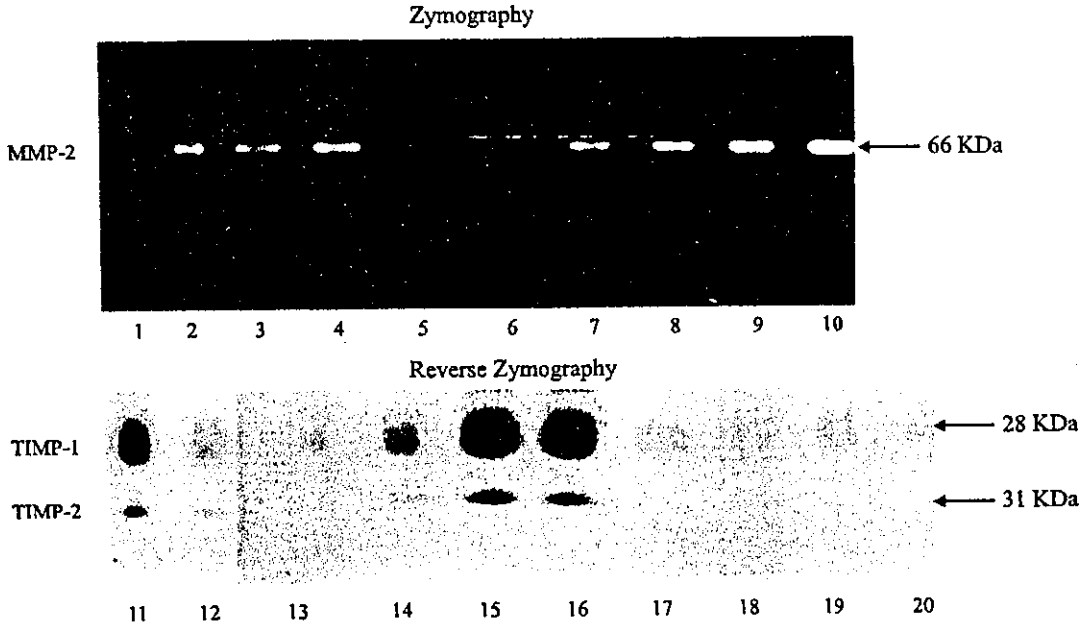

Fig. 4. Effects of SB203580 and PD98059 on MMP-2 and TIMP-1, 2 expression of TJ-9 treated hepatic stellate cells. Freshly isolated hepatic stellate cells were cultured for 4 h. The medium was replaced without TJ-9 (lanes 1, 11), with 500 μg/ml TJ-9 (lanes 2, 12), with 500 μg/ml TJ-9 plus 0.01 μM SB203580 (lanes 3, 13), with 500 μg/ml TJ-9 plus 0.1 μM SB203580 (lanes 4, 14), with 500 μg/ml TJ-9 plus 1 μM SB203580 (lanes 5, 15) with 500 μg/ml TJ-9 plus 10 μM SB203580 (lanes 6, 16), with 500 μg/ml TJ-9 plus 0.05 μM PD98059 (lanes 7, 17), with 500 μg/ml TJ-9 plus 0.5 μM PD98059 (lanes 8, 18), with 500 μg/ml TJ-9 plus 5 μM PD98059 (lanes 9, 19), or with 500 μg/ml TJ-9 plus 50 μM PD98059 (lanes 10, 20). After 4 days, zymography and reverse zymography were performed.

The effect of SB203580 (Cuenda et al., 1995), a specific inhibitor of p38 MAP kinase activation, was examined.

The expression of MMP (MMP-13, MMP-2) mRNAs was significantly reduced (Fig. 1A, 1B [b, c], lane 3). On the other hand, the effect of TJ-9 on the expression of TIMP-1 mRNA was completely diminished by treatment of SB203580 (Fig. 1A, 1B [d], lane 3).

These results were confirmed by the zymogram and reverse zymogram shown in Fig. 4 (lanes 1-6, 11-16). SB203580 reduced the activity of MMP-2 (Fig. 5) and increased the activity of TIMP-1, 2 in a dose-dependent manner in TJ-9 treated HSCs.

To elucidate the role of the ERK pathway in the effects of TJ-9, HSCs were treated with various concentrations of PD98059, a specific inhibitor of MEK activation, which prevents activation of ERK

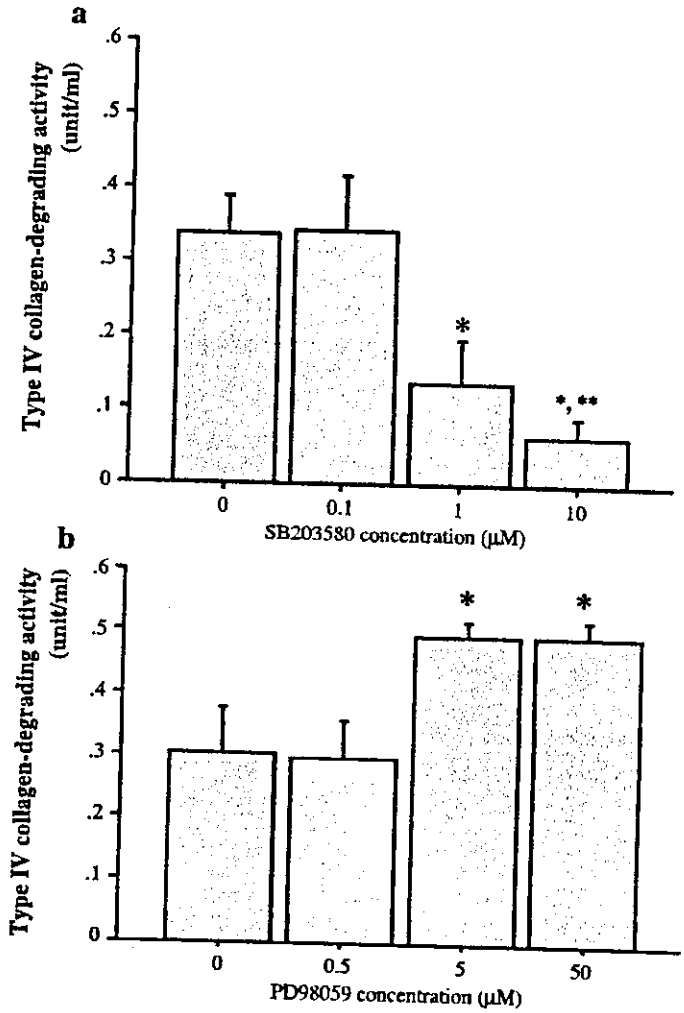

Fig. 5. Quantitative analysis of type IV collagen-degrading activity was done for the comparison of the zymography shown in Fig. 5. The results are shown as mean ± SD of 5 independent experiments. * $P<0.01$ vs 0 (control), ** $P<0.05$ vs 1 (SB203580 1 μM).

(Dudley et al., 1995). PD98059 had no noticeable effect on changes in mRNAs level but there was a mild increase in MMP-2 activity measured with zymogram (Fig. 4) and type IV collagen-degrading assay (Fig. 5).

Although SB20380 and PD980597 were dissolved in DMSO at the final concentration of 0.1%, 0.1% DMSO by itself had no effect on the activity of MMP-2 or TIMP-1, 2 (data not shown).

These results showed that the activity of p38 MAP kinase seemed to be essential to prevent TIMP-1, 2 expressions and enhance MMP-2 expression in TJ-9 treated HSCs.

Discussion

We have previously shown that TJ-9 prevents fibrosis by the inhibition of HSCs with reduced type I procollagen mRNA expression in different animal models of fibrosis due to choline-deficiency (Sakaida et at., 1998a,b), and pig serum (Shimizu et al., 1999). Also we have already reported that doses up to 1000 μg/ml of TJ-9 inhibit type I procollagen mRNA and the cell cycle of cultured stellate cell without any cytotoxicity (Kayano et al., 1998). In this study, we examined the effect of TJ-9 on the balance between MMP-2 and TIMP-1,2 with cultured HSCs.

First we showed that 500 μg/ml of TJ-9 markedly reduced TIMP-1 mRNA expression with enhanced mRNA expression of MMP-2. We choose this dose because 500 μg/ml of TJ-9 was most effective for the prevention of type I procollagen mRNA expression, as reported previously (Kayano et al., 1998). Then activity of MMP-2 and TIMPs was examined by zymogram or reverse zymogram. The reverse zymogram clearly indicated that TJ-9 reduced TIMP-1, 2 activities in a dose-dependent manner up to 500 μg/ml, with enhanced MMP-2 activity (Figs. 2, 3). The effect of TJ-9 on MMP-2 activity was also quantitatively examined by a type IV collagen degrading assay showing the peak value at the concentration of 500 μg/ml.

Secondary, the effects of TJ-9, i.e. the inhibition of TIMP-1 expression and induction of MMP-2 expression, were completely abolished by the selective p38 MAP kinase inhibitor SB203580 in a dose-dependent manner, indicating that p38 MAP kinase activity was essential for reduction of TIMP-1 expression and induction of MMP-2 expression.

In contrast, inhibition of the ERK pathway by PD98059, a selective inhibitor of MEK activation, caused only slight enhancement of MMP-2 activity without affecting mRNAs level in HSCs treated with TJ-9 (500 μg/ml).

Our findings are consistent with the recent works reporting that p38 MAP kinase may be closely related with the expression of MMP-2 in human endothelial cell (Lee et al., 2000) or the MMP-9 (Underwood et al., 2000; Simon et al., 1998) or the MMP-13 (Ravanti et al., 1999a,b) expression in different cells other than HSCs. Eberhardt W et al (Eberhardt et al., 2000) reported that IL-1β induced MMP-9 and TIMP-1 expressions in rat glomerular mesangial cells with increased activity of MPAKs cascades (ERK, SAPK/JNK and p38 MAP kinase) leading to increased activity of NF-κB and AP1 and Solis-Herruzo JA et al (Solis-Herruzo et al., 1999) also indicated that interleukin-6 induced MMP-13 and TIMP-1 expressions in rat fibroblast through stimulation of AP1. Both reports found that either MMP-9 or MMP-13 expression paralleled with the expression of TIMP-1.

It has been reported (Benyon et al., 1999)that activated hepatic stellate cells increase MMP-2 and TIMP-2 expression in a time-dependent manner on culture dishes until 7 days and that the increase in MMP-2 expression of stellate cells is responsible for degrading the normal basement

membrane, not type I collagen. Thus a question may be raised as to whether the increase in MMP-2 expression with TJ-9 treatment can be pro-fibrogenic in terms of stellate cell activation. However, TJ-9 also induced MMP-13 mRNA expression, which is responsible for degrading interstitial collagens, e.g. type I collagen. It is also known (Iredale et al., 1996) that enhanced TIMP-1,2 expresssion relative to interstitial collagenase (MMP-1/MMP-13) expression leads to the accumulation of collagens (fibrogenesis) and the reduction of TIMP-1,2 expression relative to interstitial collagenase (MMP-1/MMP-13) expression, even weak expression, can lead to the resolution of liver fibrosis (fibrolysis).

Kato et al. (Kato et al., 1998) previously reported opposite result. The treatment of melanoma cells with TJ-9 (400 μg/ml), a dose similar to that used here, showed the downregulation of MMP-2 and upregulation of TIMP-1. Studies of different cell types, e.g. malignant cells or benign cells, may reconcile these different results but further studies using other cells are necessary.

Current studies reported that an IL-10 associated silencer element (HTS) (5′ -CCACTGGCC-CATCGTATAT-3′) (-1284 to -1266 bp) in the 5′ promoter region of the TIMP-1 gene functions as a negative regulatory element that controls TIMP-1 expression (Wang et al., 1998) and upstream TIMP-1 element-1 (UTE-1) is essential for transcriptional activity of the human TIMP-1 promoter (Trim et al., 2000).

On the other hand, the promoter for the MMP-2 gene contains an AP-2 site and GC boxes (Benbow and Brinckerhoff, 1997). Chen et al. reported that MAP kinase pathways utilize a GC box to regulate gene transcription in rat HSC (Chen and Davis, 1999). It has also been reported that the MAP kinase signaling cascade stimulates Sp1 binding (Merchant et al., 1999).

But further examinations are necessary to clarify the exact relationships and cross-talk of MAPKs cascades in the expression of MMPs and TIMPs.

In conclusion, the results of this study show that TJ-9 treatment leading to upregulatation of MMP-2, 13 production and downregulation of TIMP-1,2 production. The manipulation of the balance of TIMPs and MMPs may provide new insights for the treatment of liver fibrosis.

Acknowledgements

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Proteomic profiling of proteins decreased in hepatocellular carcinoma from patients infected with hepatitis C virus

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Hepatocellular carcinoma (HCC) is a major cause of death in Japan. It has been suggested that hepatitis C virus (HCV) plays an important role in hepatocarcinogenesis, because of high incidence among the patients. To understand the mechanism of hepatocarcinogenesis after HCV infection, we performed a comparative study on the protein profiles between tumorous and nontumorous specimens from the patients infected with HCV by means of two-dimensional electrophoresis. Eleven spots were decreased in HCC tissues from over 50% of the patients. Eight proteins out of 11 spots were identified using peptide mass fingerprinting with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry. These proteins were liver type aldolase, tropomyosin β-chain, ketohexokinase, enoyl-CoA hydratase, albumin, smoothelin, ferritin light chain, and arginase 1. The intensity of enoyl-CoA hydratase, tropomyosin β-chain, ketohexokinase, liver type aldolase, and arginase 1 was significantly different ($p < 0.05$). The decrease of 8 proteins was characteristic in HCC. We will discuss the implication of these proteins for the loss of function of hepatocytes and for the possibility of carcinogenesis of HCV-related HCC.

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1 Introduction

Hepatocellular carcinoma (HCC) can be caused by chronic infection of hepatitis B or C virus. Particularly, in Japan most patients of HCC are hepatitis C virus (HCV)-positive. Accordingly, HCV may play an important role in hepatocarcinogenesis. That is, HCV carriers proceed to chronic hepatitis, to liver cirrhosis, and to HCC after incubation periods of 15, 25, and 30 years in average, respectively [1, 2]. HCC-specific markers, if present, are reliable for its early detection and for better understanding hepatocarcinogenesis. From this aspect, search for HCC-specific transcripts has been performed by means of cDNA microarray [3–10]. Iizuka *et al.* [5] reported that many genes for detoxification and immune response were upregulated in HCV-related HCC. However, expression of genes and proteins are not always uniform. Therefore, recently many studies of proteome were reported for understanding of mechanism of diseases by two-dimensional gel electrophoresis (2-DE). HCC was no exception [11–14]. Tannapfel *et al.* [15] showed that 5 proteins (insulin growth factor II, a disintegrin and metalloproteases, signal transducers and activators of transcription 3, suppressors of cytokine signaling 3, and cyclin D1) was significantly upregulated and 4 proteins (collagen 1, SMAD 4, fragile histidine triad, and suppressors of cytokine signaling 1) were downregulated in HCC by protein

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Abbreviations: HBs-Ag, hepatitis B surface antigen; **HBV**, hepatitis B virus; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **HCV-Ab**, anti-hepatitis C virus antibody

microarrays. We tried to analyze proteomics using HCV-positive HCC specimens in comparison with the surroundings. We already reported the expression of heat shock protein 70 family members increased in HCV-related HCC tissues [16].

2 Materials and methods

2.1 Tissue specimens

We examined tumorous and paired nontumorous liver specimens from 20 patients who had undergone partial hepatectomy for HCC at the Yamaguchi University Hospital between 1998 and 2000. All were positive for anti-HCV antibody (HCV-Ab) and negative for hepatitis B surface antigen (HBs-Ag). Four patient 's HCCs were well differentiated, 15 were moderately differentiated, and the last one was poorly differentiated. Twelve out of the 20 nontumorous tissues indicated cirrhotic liver.

2.2 Sample preparation

Resected liver tissues which were immediately frozen in liquid nitrogen and stored at −80°C were disrupted in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 50 mM Tris, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 μg/mL leupeptin, 10 μg/mL aprotinin) using a Potter type homogenizer with a Teflon tip at 4°C for 1 h. The lysate was separated by centrifugation at 15 000 × *g* for 30 min to yield supernatant that was stored at −80°C.

2.3 Two-dimensional gel electrophoresis (2-DE)

The supernatant from liver tissues (300 μg) was applied to Immobiline dry strips (pH 3–10, 7 cm; Amersham Pharmacia Biotechnology, Uppsala, Sweden) in a total volume of 125 μL containing 8 M urea, 2% CHAPS and 0.5% IPG buffer (Amersham Pharmacia Biotechnology) and 2.8 mg/mL dithiothreitol (DTT). After rehydration for 14 h, proteins were separated by isoelectrofocusing (IEF) at 20°C and 50 μA/strip with the following linear voltage increases: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. The strips were then equilibrated twice in 50 mM Tris containing 6 M urea, 30% glycerol and 2% sodium dodecyl sulfate (SDS) for 10 min. Dithiothreitol was then added, followed by iodoacetamide. The second dimension proceeded on 12.5% nongradient SDS-polyacrylamide gels (24.5 cm × 11 cm: SDS-PAGE) at two steps: 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min in a Multiphor horizontal electrophoresis unit (Amersham Pharmacia Biotechnology). Separated protein spots were fixed and stained on the gel with 30% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R-250 (CBB) overnight. The gel was destained with 30% methanol and 10% acetic acid for 30 min, and then with 7% acetic acid until the stain of background was clear and colorless.

2.4 Image analysis

The positions of the protein spots on tumorous and nontumorous tissues were recorded using an Agfa ARCUS 1200™ image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and analyzed with PDQUEST computer software Ver. 7.1 (Bio-Rad Laboratories, Hercules, CA, USA). Spots stained at different intensities were excised from the gels and identified by peptide mass fingerprinting with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS).

2.5 In-gel digestion

The CBB dye was removed by rinsing twice in 60% methanol containing 50 mM ammonium bicarbonate and 5 mM DTT for each 15 min, and twice in 50% acetonitrile containing 50 mM ammonium bicarbonate and 5 mM DTT for each 7 min. The gel piece was dehydrated in 100% acetonitrile, and then reswollen with an in-gel digestion reagent containing 10 μg/mL sequencing-grade trypsin (Promega, Madison, WI, USA) in 30% acetonitrile of 50 mM ammonium bicarbonate and 5 mM DTT. The in-gel digestion was performed overnight at 30°C.

2.6 Peptide mass fingerprinting

After in-gel digestion, 1 μL of the reaction mixture was removed and mixed with 1 μL of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 40% methanol, 0.1% trifluoroacetic acid) on a MALDI target plate. MALDI-TOF-MS for peptide mass fingerprinting was performed on AXIMA-CFR mass spectrometer (Shimadzu Biotech, Kyoto, Japan) in reflectron mode. The MS-Fit database search engine in the Protein Prospector web site (http://prospector.ucsf.edu/) was used for protein identification.

2.7 Immunoblot analysis

Samples of 7 μg were separated by SDS-PAGE at 15 mA. After SDS-PAGE, the fractionated proteins were transferred electrophoretically to a PVDF membrane (Immobilon; Millipore, Bedford, MA, USA) and blocked overnight at 4°C with TBS containing 5% skim milk. Primary antibodies used were anti-aldolase B polyclonal antibody (1:100) and anti-arginase 1 polyclonal antibody (1:100)

(all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). For each, membranes were incubated for 1 h, washed four times with TBS containing 0.05% Tween 20, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2000) (ICN Pharmaceuticals, Aurora, OH, USA), and developed with a chemiluminescence reagent (ECL; Amersham Pharmacia Biotechnology).

3 Results

We compared the differential expression of proteins by 2-DE of paired tumorous and nontumorous liver tissues from 20 patients with HCC. Staining with CBB R-250 detected about 480 protein spots (Fig. 1). Eleven of these were decreased in tumorous tissues from over 50% of the patients. Peptide mass fingerprinting with MALDI-TOF-MS identified ketohexokinase, arginase 1, liver type aldolase, enoyl-CoA hydrase, tropomyosin β-chain, albumin, smoothelin, and ferritin light chain (Fig. 2). Information about the 11 spots and the 8 identified proteins are summarized in Table 1. The expression of arginase 1 was decreased in 95% of HCC patients. Liver-type aldolase was decreased in 85%, tropomyosin β-chain in 80%, ketohexokinase, enoyl-CoA hydrase and albumin in 70%, smoothelin in 65%, and ferritin light chain in tumorous tissues from 60% patients. We performed pairwise *t*-tests between the means of spot intensity of tumorous tissues and nontumorous tissues. The intensity of enoyl-CoA hydratase, toropomyosin β-chain, ketohexokinase, liver type aldolase, and arginase 1 significantly differed between tumorous and nontumorous tissues (Fig. 3). The

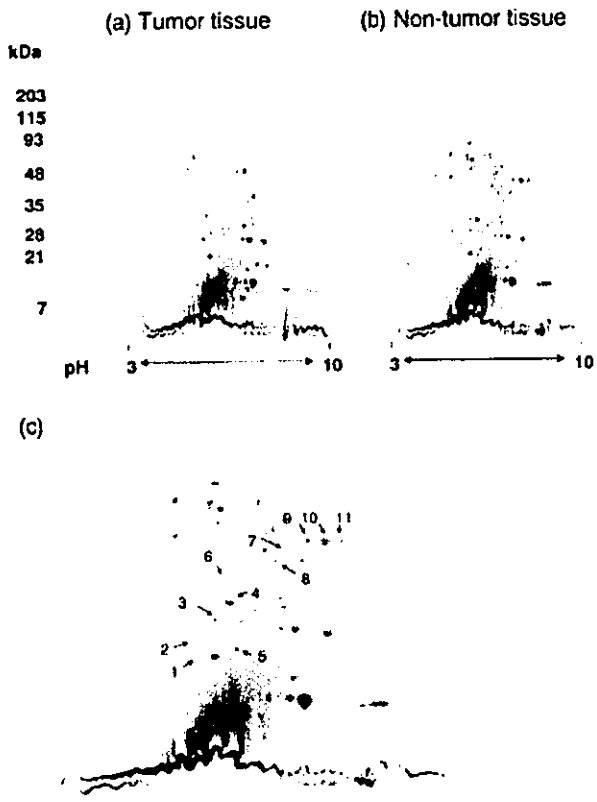

Figure 1. Two-dimensional gel electrophoresis map of human liver tissue. (a) Tumorous tissue, (b) nontumorous tissue of a HCC patient infected with HCV. Proteins (300 μg) were separated on 12.5% nongradient polyacrylamide gels and stained with CBB. (c) Enlarged 2-DE map from (b); protein spots are numbered from 1 to 11.

Table 1. Protein spots that decreased in HCC tissues

Spot No.[a]	Accession No.	Protein	Number of patients[b] (%)	Spot intensity (average ± SD)		Ratio of spot intensity[c] (% average ± SD)
				Tumorous tissue	Nontumorous tissue	
1	P53814	Smoothelin	13 (65%)	2 466.2 ± 3 333.6	2 872.8 ± 1 688.4	108.57 ± 115.09
2	P02792	Ferritin light chain	12 (60%)	2 601.1 ± 2 590.2	2 936.4 ± 2 408.1	286.52 ± 521.40
3	P02768	Albumin	14 (70%)	25 635.1 ± 77 312.6	12 585.5 ± 11 585.3	189.58 ± 401.46
4	P30084	Enoyl-CoA hydratase	14 (70%)	13 110.9 ± 15 939.1	27 113.4 ± 19 645.5	86.66 ± 112.12
5	P07951	Tropomyosin β-chain	16 (80%)	1 780.7 ± 1 423.4	4 984.9 ± 5 349.2	59.20 ± 50.69
6	P50053	Ketohexokinase	14 (70%)	1 842.2 ± 1 534.1	3 407.6 ± 1 951.5	107.36 ± 181.62
7	P05089	Arginase 1	19 (95%)	2 394.0 ± 2 331.7	7 460.4 ± 5 742.2	99.36 ± 299.39
8	P05062	Liver-type aldolase	17 (85%)	2 321.1 ± 1 766.5	5 822.9 ± 6 591.3	104.30 ± 195.60
9	P05062	Liver-type aldolase	17 (85%)	4 705.5 ± 5 199.0	20 619.2 ± 26 706.9	48.73 ± 67.16
10	P05062	Liver-type aldolase	18 (90%)	6 122.1 ± 3 997.5	60 282.2 ± 161 480.1	57.40 ± 107.92
11	P05062	Liver-type aldolase	17 (85%)	22 109.5 ± 19 933.8	113 699.8 ± 164 866.2	61.97 ± 105.57

a) Spot numbers correspond to that in Fig. 1c.
b) Number of patients in which protein expression decreased in tumorous tissues
c) Percentage of spot intensity of tumorous to nontumorous tissues calculated for each sample pair

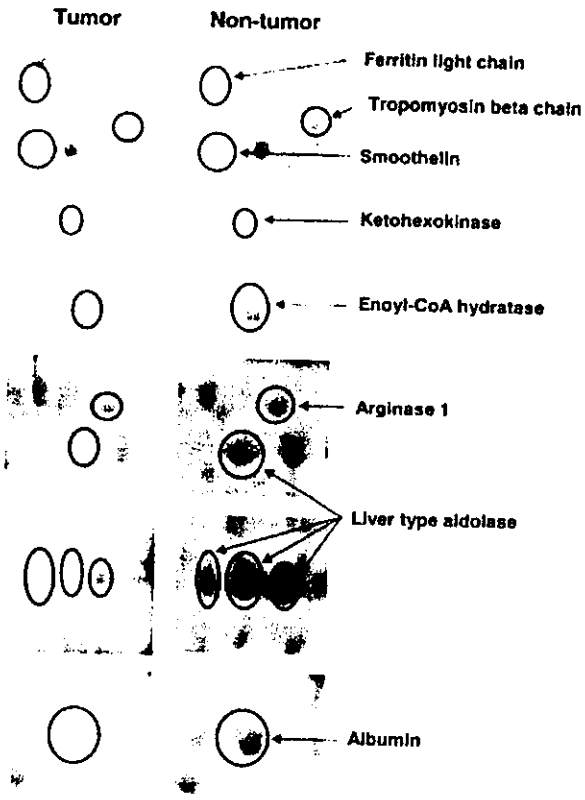

Figure 2. Downregulated expression of 11 spots and 8 proteins in HCC. Spots of all 11 proteins that decreased in tumorous tissues include smoothelin, ferritin light chain, tropomyosin β-chain, albumin, ketohexokinase, enoyl-CoA hydratase, liver type aldolase, and arginase 1.

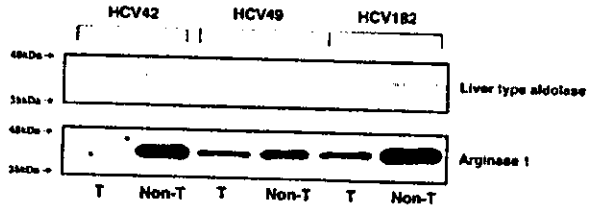

Figure 4. Immunoblot analysis of liver-type aldolase (aldolase B) and arginase 1. Expression of the two proteins was confirmed and the intensity of each band was less in tumorous tissues (T) than in nontumorous tissues (Non-T). Three samples (HCV42, HCV49, and HCV182) from 20 were shown. The molecular weight markers are indicated by arrows.

expression of two proteins among five, liver type aldolase and arginase 1, whose antibodies were available, was confirmed by immunoblot analysis. The intensity of each band was less in tumorous tissues than in nontumorous tissues (Fig. 4).

4 Discussion

There were some previous proteomic studies for HCC, and the etiologies of their HCCs were abundant. Many of them were hepatitis B virus (HBV)-related HCCs [11–14]. However, all of the patients of our study were HCV-Ab-positive and HBs-Ag-negative. Since the hepatocarcinogenetic mechanism of HCV may be different from HBV, we need to construct several databases of HBV- and HCV-related HCCs. We found that the decrease of keto-

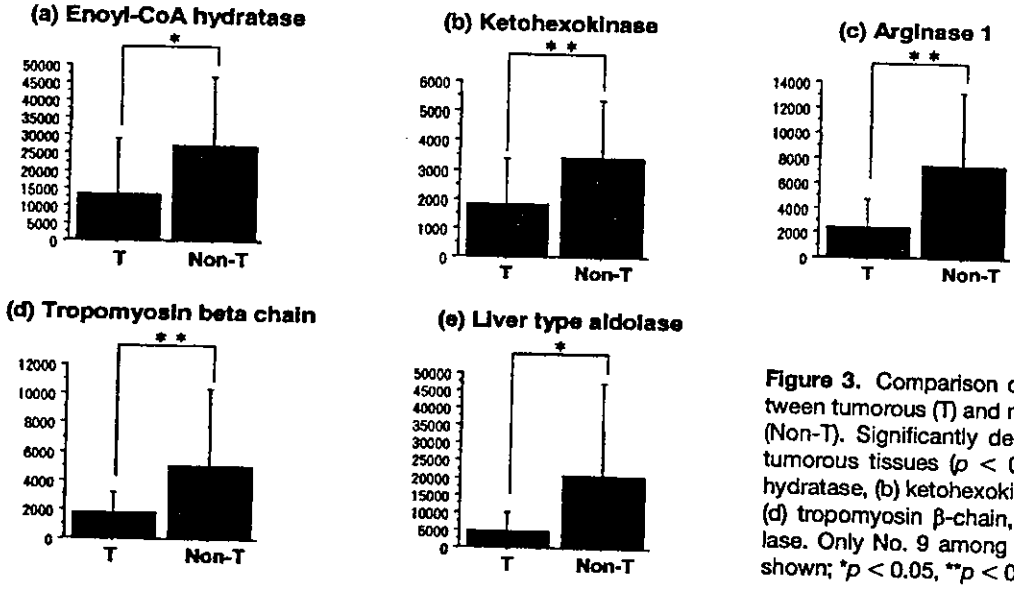

Figure 3. Comparison of spot intensity between tumorous (T) and nontumorous tissues (Non-T). Significantly decreased proteins in tumorous tissues ($p < 0.05$): (a) enoyl-CoA hydratase, (b) ketohexokinase, (c) arginase 1, (d) tropomyosin β-chain, (e) liver type aldolase. Only No. 9 among 4 aldolase spots is shown; $^{*}p < 0.05$, $^{**}p < 0.01$.

hexokinase, ferritin light chain, and enzymes involved in the glycolysis and mitochondrial β-oxidation pathways concurs with other human HCC proteomic studies. We discovered also the decrease of smoothelin and tropomyosin β-chain. The proteins which we detected in this study are classified as metabolic enzymes and cytoskeletal proteins. Smoothelin and tropomyosin β-chain belong to the latter. Smoothelin is a cytoskeletal protein existing in visceral and vascular smooth muscle cells [17] as 59 kDa smoothelin-A and 110 kDa smoothelin-B [18, 19]. Rensen *et al.* [20] could not detect smoothelin in normal liver by Western blotting. However, we could detect by 2-DE smoothelin on both gels of tumorous and nontumorous tissues from patients infected with HCV. The use of samples infected with HCV may cause this discrepancy. Hepatic stellate cells (HSCs) have fatty droplets including vitamin A and regulate the sinusoidal microcirculation in normal liver [21, 22]. Once liver tissues are injured, HSCs are activated and change their structure and function. Activated HSCs transform themselves into the myofibroblast and lead to overexpression of α-smooth muscle actin [23, 24]. These data indicated that the source of smoothelin might be not hepatocytes but intermediate cells. Although smoothelin may not be specific to cancer cells, interaction between cancer cells and intermediate cells is important. In accordance with progression of hepatocarcinogenesis, cancer tissues destroy hepatic cords. Therefore, smoothelin may become a marker of HCC progression.

Tropomyosin binds to actin filaments in muscle as well as nonmuscle cells, and regulates muscle contraction in conjunction with troponin. Tropomyosin is a heterodimer of α- and β-chains. In nonmuscle cells such as hepatocytes, tropomyosin plays a role of stabilization of actin filaments [25, 26]. It was reported that tropomyosin suppressed the transformation of phenotypes in nonmuscle cells [27–29]. In previous proteomic studies using 2-DE, the expression of tropomyosin-2 decreased in human ovarian cancer tissues [30] and the expression of tropomyosin β-chain decreased in human prostate cancer tissues [31]. In addition, the expression of tropomyosin decreased in several cancer tissues [30–33].

In metabolic enzymes, the expression of liver-type aldolase considerably decreased in our tumorous tissues, and this decrease has never been reported in previous proteomic studies on human HCCs. Aldolase performs the sixth step of glycolysis. Aldolase has three isozymes. Aldolase A exists in muscle, aldolase B in liver, and aldolase C in brain [34]. Weinhouse *et al.* [35] have shown that liver-type aldolase was replaced by nonliver type aldolase in poorly differentiated rat liver tumors. Kinoshita *et al.* [36] have reported that the mRNA of aldolase B was downexpressed in 18 of 20 HCC patients, and Kimura *et al.* [37] found that the expression level of aldolase B was low and aldolase A was high in hepatoma cell lines. Salvatore *et al.* [38] have shown that the mRNA expression of aldolase A in HCC tissues was higher than that in surrounding cirrhotic tissues. Aldolase A is exclusively expressed in the fetal liver [39]. As the expression of carcinoembryonic antigen (CEA) or α-fetoprotein (AFP) which are expressed during embryonic or fetal terms increase in cancer tissues, the expression of aldolase A might increase in HCC tissues by replacing aldolase B. We found the spots of albumin and smoothelin at different positions from those predicted by the *pI* value and molecular weight on both gels of tumorous and nontumorous tissues. These results might be caused by post-translational modifications or mutation by HCV infection. These changes could be characteristic of HCV-related HCCs.

In this study, we performed the first proteomic analysis of human HCV-related HCCs. A protein expression database constructed by reference to our study may be useful for the discovery of new tumor markers and for producing new diagnostic techniques.

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Transplantation of Bone Marrow Cells Reduces CCl_4-Induced Liver Fibrosis in Mice

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We investigated the effect of bone marrow cell (BMC) transplantation on established liver fibrosis. BMCs of green fluorescent protein (GFP) mice were transplanted into 4-week carbon tetrachloride (CCl_4)–treated C57BL6 mice through the tail vein, and the mice were treated for 4 more weeks with CCl_4 (total, 8 weeks). Sirius red and GFP staining clearly indicated migrated BMCs existing along with fibers, with strong expression of matrix metalloproteinase (MMP)-9 shown by anti–MMP-9 antibodies and *in situ* hybridization. Double fluorescent immunohistochemistry showed the expression of MMP-9 on the GFP-positive cell surface. Film *in situ* zymographic analysis revealed strong gelatinolytic activity in the periportal area coinciding with the location of MMP-9–positive BMCs. Four weeks after BMC transplantation, mice had significantly reduced liver fibrosis, as assessed by hydroxyproline content of the livers, compared to that of mice treated with CCl_4 alone. Subpopulation of Liv8-negative BMCs was responsible for this fibrolytic effect. In conclusion, mice with BMC transplants with continuous CCl_4 injection had reduced liver fibrosis and a significantly improved survival rate after BMC transplantation compared with mice treated with CCl_4 alone. This finding introduces a new concept for the therapy of liver fibrosis. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2004;40: 1304–1311.)

Recent reports have shown the capacity of the bone marrow cell (BMC) to differentiate into a variety of non-hematopoietic cell lineages.[1-5] These results indicate that the BMC is an attractive cell source for regenerative medicine compared with tissue-specific stem cells.[6] The capacity of the BMC to differentiate into hepatocytes and intestinal cells has been shown by Y-chromosome detection in autopsy analysis of human female recipients of BMCs from male donors.[7,8] Although Lagasse et al. reported that purified hematopoietic stem cells could differentiate into hepatocytes using a fumarylacetate hydrase-deficient model,[5] Wagers et al. showed little evidence of plasticity in adult hematopoietic stem cells.[9] Thus, although there is still controversy about which part of BMCs can differentiate into hepatocytes, the BMC seems to have the plasticity to differentiate into such cells. From the point of view of therapy, one of the targets of liver disease for BMC transplantation is liver cirrhosis with chronic liver failure. This is an unphysiological condition with excessive deposition of extracellular matrix and a relative lack of parenchymal cells (hepatocytes). Even if BMC transplantation is successful in supplying parenchymal cells, the fate of the extracellular matrix under these conditions is unknown. The present study clearly shows that transplanted BMCs reduce (degrade) carbon tetrachloride (CCl_4)-induced liver fibrosis with a significantly improved survival rate.

Materials and Methods

Mice. GFP-transgenic mice (TgN(β-act-EGFP)Osb) were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan).[10] C57BL6 female mice were purchased from Japan SLC

Abbreviations: BMC, bone marrow cell; CCl4, carbon tetrachloride; GFP, green fluorescent protein; PBS, phosphate-buffered saline; IgG, immunoglobulin G; MMP, matrix metalloproteinase; NGS, normal goat serum; NRS, normal rabbit serum; DIG, digoxigenin.

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(Shizuoka, Japan). Mice were properly anesthetized during experiments.

Experimental Protocol. Six-week-old female C57BL6 mice were treated with 1 mL/kg CCl_4 dissolved in olive oil (1:1) twice a week for 4 weeks. One day (24 hours) after the eighth injection of CCl_4, 1×10^5 green fluorescent protein (GFP)-positive BMCs or sorted Liv8-positive or Liv8-negative BMCs (1×10^5 cells) or same volume of saline as a control (described also as mice treated with CCl_4 alone) were injected into the tail vein as described previously.[11,12] Mice continued to be treated with CCl_4. After 1, 2, 3, or 4 weeks, mice were then sacrificed to assess the extent of liver fibrosis. For examination of the survival rate, mice were treated with CCl_4 for 4 weeks and divided into 2 groups (15 mice each) with bone marrow transplantation or the same volume of saline injection. All mice were then treated with CCl_4 for a further 25 weeks.

BMC Preparation. For BMC isolation, GFP-transgenic mice (TgN(β-act-EGFP)Osb) (6 weeks old) were killed by cervical dislocation and the limbs removed. GFP-positive BMCs were flushed with Dulbecco's Modified Eagle medium (DMEM) culture medium with 10% fetal bovine serum (FBS) from the medullary cavities of tibias and femurs using a 25-G needle.

Production of Rat Monoclonal Antibody, Liv8. Eight-week old WKY/NCrj female rats were immunized in the hind footpads with 100 μg of E11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 mL). Anti-Liv8 antibodies were raised according to a previously described protocol.[13]

Fluorescence-Activated Cell Sorter Analysis of Fetal Liver Cells and BMCs Using Anti-Liv8 Antibody. Prepared mouse fetal liver cells (E11.5) and adult BMCs were reacted with biotin-conjugated anti-Liv8 antibody,[12] phycoerythrin-conjugated rat anti-CD45 (Becton Dickinson Bioscience, San Jose, CA), fluorescein isothiocyanate-conjugated anti–c-kit (Becton Dickinson Bioscience), phycoerythrin-conjugated anti–Thy 1 (Becton Dickinson Bioscience), and fluorescein isothiocyanate-conjugated anti-B220 antibodies (Becton Dickinson Bioscience) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the tube for 30 to 40 minutes at 4°C. Following the incubation with the first antibody, the cells were washed twice by 0.02 mol/L phosphate-buffered saline (PBS) and centrifuged at 500*g* for 5 minutes. Labeled cells were then reacted to allophycocyanin-conjugated streptavidin (Becton Dickinson Bioscience) at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the tube for 30 to 40 minutes at 4°C. After that, these were washed out once with 0.02 mol/L PBS and centrifuged at 500*g* for 5 minutes. The labeled cells were analyzed using FACS Calibur (Becton Dickinson Bioscience).

Preparation of Liv8-positive and Liv8-negative BMCs Liv8-positive and Liv8-negative BMCs were prepared as described previously.[12] Briefly, prepared BMCs were reacted to rat anti-Liv8 immunoglobulin G (IgG) antibody at the rate of 1 μg per 10^6 total cells, mixed well, and incubated in the tube for 30 minutes at 4°C. Cells were then washed twice by 0.02 mol/L PBS and centrifuged at 500*g* for 5 minutes. Cells were labeled with rat anti-Liv8 IgG antibody by reacting with goat anti-rat IgG MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at the rate of 20 μL per 10^7 total cells, mixed well, and incubated for 20 minutes at 4°C. Labeled cells were washed once by 0.02 mol/L PBS and centrifuged at 500*g* for 5 minutes. These cells were separated into Liv8-positive cells or Liv8-negative cells by the autoMACS magnetic cell sorting system (Miltenyi Biotec GmbH) for 10 minutes per tube.

Tissue Preparation and Immunohistochemistry. The liver was perfused via the heart with 4% paraformaldehyde to flush out blood cells and incubated with 4% paraformaldehyde overnight. Tissues were then soaked in 30% sucrose for 3 days. Tissues were frozen with liquid nitrogen to prepare for sectioning with a cryostat for immunohistochemistry.

Cells expressing GFP and matrix metalloproteinase (MMP)-9 (or α-smooth muscle actin) were analyzed by both fluorescent microscopy and conventional immunohistochemistry using anti-GFP, anti–MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti–α-smooth muscle actin antibodies (Sigma-Aldrich, St. Louis, MO). Tissues were soaked in 0.3% Triton X-100 with 0.05% normal goat serum (NGS) (Chemicon, Temecula, CA) or normal rabbit serum (NRS) (Chemicon) in PBS overnight. The next day, the tissues were put in 500 mL of 10% NGS or NRS in 0.3% Triton X-100 of PBS for 2 hours, then washed with 0.3% Triton X-100 with 0.05% NGS or NRS in PBS for 10 minutes. We soaked the tissues in 1.5% H_2O_2 in 50% methanol with distilled water for 2 hours. The tissues were then washed in 0.3% Triton X-100 with 0.05% NGS or NRS in PBS. Sections were incubated with anti-GFP and anti–MMP-9 (ICN Pharmaceuticals Inc., Kanagawa, Japan) antibodies. Anti–biotin-conjugated anti-goat IgG, anti-rabbit IgG, biotin-conjugated rabbit anti-goat IgG, and biotin-conjugated rabbit anti-mouse IgG were purchased from Dako Japan (Kyoto, Japan) and used as the secondary antibodies. PAP-goat (B0157), PAP-mouse (B0650), and PAP-rabbit (Z0113) polyclonal antibodies (Dako Japan) were used as third antibodies.

For fluorescent immunohistochemistry, we used Alexa Fluor R 488 and 568 donkey anti–goat- or anti–rabbit-