Table 1
Reproducibility of the elastometry (kPa). Two data of the same case were measured 3 months after the initial measurement

Cases	Before (kPa)	Three months later (kPa)
A	10.4	12.6
B	12.5	12.5
C	14.3	19.0
D	17.4	13.1
E	46.7	48.2
F	10.0	9.7
G	12.9	12.9
H	10.2	13.9
I	10.4	10.3
J	11.9	11.9
K	14.3	10.1
L	16.7	17.4
M	6.9	8.8
N	9.0	9.2

unexpected cause of liver diseases, such as metabolic diseases [16,23–26]. It is also useful for identifying early fibrosis that may allow earlier intervention well before progression to cirrhosis, and especially in the natural history of chronic hepatitis C, the establishment of fibrotic stage may be helpful for early detection of hepatocellular carcinoma [26–28]. In case of the patient with chronic hepatitis C in older age such as more than 70, and the liver is slight fibrotic, he or she has no need for anti-viral therapy. Another benefit of liver biopsy is providing motivation of the patient for having treatment early or lifestyle change. On the other hand, although risks can be possibly reduced by operator experience and using an ultrasound guidance, several known risks of obtaining the tissue, such as pain, bleeding, pneumothorax, hemothorax, bile peritonitis, hemobilia, puncture of kidney and intestine, infection, anxiety and even death, do not seem to be entirely avoidable [16–18,29]. The present study showed the non-invasiveness, no risk and reliability of the elastometry in the evaluating hepatic fibrosis, and this procedure can be substituted for liver biopsy in the risky patient. The most useful indication of this measurement may be the sequential changes of hepatic fibrosis in follow-up of the same patient with or without treatment.

A reliable way to determine the presence of advanced fibrosis with non-invasive procedures is needed in chronic hepatitis C. The combination of biochemical markers and clinical and imaging findings can identify much advanced fibrosis [27]. A high aspartate/alanine aminotransferase ratio or low platelet count in patients with chronic hepatitis C has a good predictive value of advanced fibrosis [30]. This study also suggested that platelet counts correlated with the stage of hepatic fibrosis, and the result suggests that we can estimate a patient who becomes cirrhosis when his or her platelet counts decreases under $100 \times 10^3\ \mu l^{-1}$. But the deviation is so large that the cross-sectional approach with platelet counts cannot directly predict hepatic fibrosis. In several European countries, a more complexed formula of tests with γ-globulin, γ-glutamyltranspeptidase, total bilirubin, α2-macroglobulin, haptoglobin, and apolipoprotein A1 achieved to identify patients with and without advanced fibrosis [31]. But this test could categolize patients into two groups with accuracy of 46%. Compared to the value of platelet counts, and also to the value of serum fibrosis markers in the present study, the result of elastometry seemed to be cross-sectionally more reliable, because a deviation was not so large. From this study, we may assess the fibrotic stage of the patient with chronic hepatitis C by elastometry. We can assess as F1 if the result of elastometry is around 5 kPa, as F2 if the result is around 10 kPa, and as F3 if the result is around 15 kPa. If the data were more than 20 kPa, the liver may be in F4 stage, according to the new Inuyama classification [22].

It is thought that the elasticity measured by this device may be influenced by the fibrosis patterns, such as a difference between thick fibrosis and fine fibrosis, that may vary even if the total extracellular matrix content is same. Therefore, the most indicatable case of this device may be the follow-up of the same individual. The elasticity of the liver might be different in patients with chronic viral infection and alcoholic liver diseases, because the intrahepatic fibrotic patterns are different. This is because we investigated only the group of patients with chronic hepatitis C. Liver fibrosis is a complex process, involving injury to liver cells and inflammatory response and scaring [1,6]. In response to liver injury, hepatic stellate cells are activated and proliferate [32,33], resulting in secretion of cytokines [2,3], matrix proteins [34] and their counterparts [35]. The balance of their dynamisms may determine the pattern of fibrotic change in the liver. There are many potential therapies, including antioxidants [36–38], cytokine modulators [35,39–41], anti-inflammatory drugs [42–45], inhibitors of collagen deposition, and inhibitors of stellate cell activation [5,46]. The most primary therapy to reverse fibrosis is elimination or control of the injuring agent such as virus, alcohol and iron, and this therapy successfully reverses cirrhosis [12,47–49]. Although it is not sure that genetic treatment that can stop or reverse fibrosis when the primary pathogenetic agent cannot be eliminated, the elastometry seems to be a good marker for assessing changes achieved by the treatment of fibrosis.

There may be many factors influencing to the measurement of velocity of a transient vibration, but little is known about this apparatus. According to the report described by Sandrin et al. [19], who first developed this apparatus, elasticity measurements were related only to the fibrosis grade and not to the activity and steatosis grades. But he also described that fat tissue may absorb or diminish low-frequent vibration, resulting in a poor signal to noise ratio that affects the elasticity measurement algorithm in patients with obesity. The low-frequency elastic waves do not propagate through liquids, indicating that elastometry is not impossible in patients with ascites. The chest wall contributes to prevent the liver from being directly compressed by the probe itself, and to give a static and plane surface for the probe

positioning. Blood flow might be another consistent factor for the measurement. We wondered that fatty liver is softer than healthy liver parenchyma, suggesting that steatosis would be expected to induce a decrease of liver elasticity. Further evaluation for the elastometory of liver fibrosis in patients with steatosis is necessary. The growing awareness of increasing prevalence of non-alcoholic fatty liver diseases made increased frequency of biopsy, because this type of disease entity has not been established until its diagnostic criteria is determined. Diehl et al. [50] demonstrated the value of liver biopsy for identifying unsuspected but treatable forms of liver disease. This report suggested that the result of liver biopsy sometimes prevents patients from receiving inappropriate treatment. Although the elastometry may assess the changes of fibrotic stage in this type of liver diseases, it is conceivable that we can exactly assess the absolute elastic degree of the liver with fatty degeneration. This is why we selected the patients whose histology showed fatty deposit less than 10% of hepatocytes in whole biopsy samples. The correlation of elastometry and fibrotic stage of the liver in patients with other liver diseases is open for discussion in future, when the data of both histology and elastometry in individuals are collected in various liver diseases.

There are several semiquantitative scores used in clinical trials and in retrospective analysis such as the Knodell [51], the Scheuer [52], the Metavir [53], the Batts–Ludwig [54], and the Ishak [55] scores in the Western countries. In Japan, new Inuyama classification has been used since the older classification was largely modified in 1996 [22]. Criteria for staging of fibrosis was follows; F0, no fibrosis; F1, fibrous portal expansion; F2, bridging fibrosis (portal-portal or portal-central linkage); F3, bridging fibrosis with lobular distortion (disorganization); F4, cirrhosis. Compared with the Metavir score, this Japanese score may includes more severe fibrosis in F2–F4 than that of the Metavir system. Since morphologic features of chronic hepatitis C are sometimes interpreted with interobserver and intraobserver variations, and also the scoring system is different between countries, quantitative evaluation of hepatic fibrosis is necessary. The elastometry will be a good quantitative indicator for hepatic fibrosis if elastometry exactly reflects the whole extracellular matrix content of the liver, and influence of fatty change and other factors for measurement is elucidated.

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Down-regulation of matrix-invasive potential of human liver cancer cells by type I interferon and a histone deacetylase inhibitor sodium butyrate

FUMIHIKO KANEKO[1,2], HIDETSUGU SAITO[1], YOSHIMASA SAITO[1], KANJI WAKABAYASHI[1], NOBUHIRO NAKAMOTO[1], SHINICHIRO TADA[1], HIDEKAZU SUZUKI[1], SATOSHI TSUNEMATSU[2], NAOKI KUMAGAI[2] and HIROMASA ISHII[1]

[1]Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582; [2]Liver Center, Department of Internal Medicine, Kitasato Institute Hospital, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan

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Abstract. We have demonstrated anti-proliferation and anti-metastasis effects of both interferon-α and a histone deacetylase inhibitor, sodium butyrate, on human liver cancer cell lines. In this study, invasive ability of human liver cancer cell lines through the matrix-coated membrane was examined and inhibitory effect of interferon-α and sodium butyrate was investigated. Among six human liver cancer cell lines, HLE and HLF showed high invasive ability using the Matrigel invasion assay. This invasion ability was significantly inhibited by pretreatment of the cells with 1000 IU/ml of interferon-α or 2 mM of sodium butyrate. Gelatin zymography and the matrix metalloproteinase-2 and -9 activity assay showed that these two cell lines produce active- and pro-matrix metalloproteinase-2 and -9, and their activity was significantly reduced by pretreatment with both agents. Real-time quantitative reverse transcription-polymerase chain reaction showed decrease in matrix metalloproteinase-1 mRNA levels by pretreatment with both agents, but mRNA levels of tissue inhibitor of matrix metalloproteinase-1 and -2 were differently modulated by interferon-α and sodium butyrate. These results suggest that interferon-α and sodium butyrate reduce a chance of invasion and metastasis of human liver cancer cells by inhibiting matrix metalloproteinase activity, although its inhibitor is differently regulated.

Correspondence to: Dr Hidetsugu Saito, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
E-mail: hsaito@sc.itc.keio.ac.jp

Key words: invasion, matrix metalloproteinase, hepatocellular carcinoma, type I interferon, sodium butyrate

Introduction

Human hepatocellular carcinoma (HCC) is one of the malignant tumors prevalent world-wide. The number of patients with HCC is still increasing in Japan, especially that caused by chronic hepatitis C virus (HCV) infection. The incidence of HCC is higher in patients with chronic hepatitis C than in those with chronic hepatitis B and non-B, non-C including alcoholic (1). Epidemiological data suggest that there is a close correlation between duration of chronic HCV infection and incidence of hepatocarcinogenesis. HCV is a ribonucleic acid (RNA) virus that is not reverse transcribed to deoxynucleic acid (DNA), and this virus does not invade into the nuclei and never achieve integration to host DNA. This virus by itself does not explain the molecular mechanism of hepatocarcinogenesis in chronic HCV infection. Genomic instability caused by HCV-related chronic inflammation may be the most important event in HCV-related carcinogenesis (2), because it takes a long time for development of HCC after initial infection of HCV and recent studies revealed that the relationship between duration of the infection, fibrosis of the liver, and HCC occurrence rate is very close (3,4), and moreover elimination of HCV resulted in reduction of HCC occurrence rate after then (5). Thus, the liver with chronic HCV infection is considered to be in a hypercarcinogenic state, the state in which genes are prone to mutation (6).

In such a hypercarcinogenic state, HCC develops all around the liver whether or not multiple HCC develops at the same time or at the different time, resulting in frequent recurrence of HCC after hepatic resection has occurred in the different regions (4). Since the high-risk situation of HCC development has been defined and we closely followed up the patients by the ultra sound and other diagnostic examinations, we can detect small HCC, <2 cm diameter. These observations, that the frequent recurrence of HCC and the detection of small HCC, lead hepatologists to treat HCC with local ablation modalities, such as percutaneous ethanol injection, microwave coagulation therapy, and radio

frequency ablation (7), unless liver transplantation is indicated. In advanced cases, transarterial chemo-embolization (TACE) is another effective therapeutic modality (8,9). These various therapeutic procedures have decreased death rate of this disease, but it is still insufficient and we have no sufficiently effective therapy for its metastasis to other organs. Recently chemo-adjuvant therapy using type I interferon (IFN) has been introduced and is shown to be effective in advanced cases (10-12). However, the contribution of this treatment to life expansion is still under investigation (13).

We have studied the chemopreventive effect of differentiation inducers for human HCC cells (14). Among them, type I IFN and sodium butyrate, one of histone deacetylase (HDAC) inhibitors, were the most effective agents (15). Type I IFN can induce apoptosis of human HCC cells (16), and if the cell is not sensitive for apoptosis (17), cell cycle arrest is induced by IFN, resulting in reduction of cell proliferation and decrease in malignant phenotypes, probably according to the genomic polymorphisms (18,19). On the other hand, butyrate is a 4-carbon short fatty acid, which is a fermented product, and an HDAC inhibitor, which changes genomic transcription epigenetically. Recent studies showed that epigenetic changes such as DNA methylation in CpG islands of some oncogenes and histone acetylation are important in hepatocarcinogenesis (20).

These two agents, IFN and butyrate, similarly reduced HCC cell proliferation with increase in albumin production and decrease in α-fetoprotein production (15,21). These also similarly reduced c-myc expression (22,23), telomerase activity (24), and a metastasis potential of HCC cells by increasing expression of cell-cell adhesion molecules E-cadherin and ß-catenin (25). Thus, these two agents may affect another malignant potential of HCC, invasion ability, but little is known about this characteristic so far. It was demonstrated that human HCC cells require matrix metallo-proteinases (MMP) activity for migration and invasion (26). In the present study, to investigate the effect of type I IFN and sodium butyrate on invasive potential of human HCC cells, the matrix-coated gel invasion assay was performed and effect of IFN and butyrate on the production of MMP-1, -2 and -9 or tissue inhibitor of MMP (TIMP)-1 and 2 from HCC cells were examined. Thus, we investigated possible chemopreventive effect of the agent and its mechanisms in human HCC.

Materials and methods

Cells. Human HCC cell lines, HLE, HLF (27), HepG2, PLC/PRF/5, HCC-T (28) and HCC-M (29) were used in this study as described elsewhere (30). HCC-T and HCC-M were cultured in RPMI-1640 (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 IU/ml of penicillin (Gibco) and 100 μg/ml of streptomycin (Gibco). HLE, HLF, HepG2 and PLC/PRF/5 were cultured in Eagle's modified essential medium (Nissui Seiyaku) supplemented with the same agents. These cells were cultured at 37˚C in the 5% CO_2 incubator and the medium was changed every two days.

Detection of apoptosis. To confirm that the treated cells were not dead, we observed morphologic aspect of the cell and further performed terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay in the same experimental condition. The procedure of TUNEL assay has already been described (31).

Cell mobility assay. Since cell mobility is concerned to cancer cell invasion and metastasis, we tried to quantitatively measure the cell mobility and evaluate the effect of IFN-α and sodium butyrate using an inverted phase-contrast microscope (Nikon TMD300, Nikon, Tokyo, Japan) equipped with a digital time-lapse image analyzing system (Aquacosmos, version 2.0, Hamamatsu Photonics, Hamamatsu, Japan). The image of the cell was digitally recorded before the application of the test drug (0 h) and every 30 min after the start of the test drug for 2 days under the cell culture conditions. Collected 97 digital images were analyzed by a digital image analyzer.

Matrigel invasion assay. In vitro invasive potential of cancer cells was assayed using the Biocoat Matrigel Invasion Chamber (32) (Becton Dickinson Labware, Bedford, MA). Human HCC cells ($5x10^4$) were seeded onto the upper chamber of double-structured matrix gel chamber with 1% bovine serum albumin (BSA, Sigma-Aldrich Japan K.K., Tokyo, Japan) and the conditioned culture supernatant of NIH/3T3 cells, which was obtained from serum-free culture, was added to the lower chamber as a chemo-attractant. In this assay system, the cancer cells dissolve the matrix gel consisting of laminin, collagen type IV, heparan sulfate, proteoglycan, entactin and invade into 8 μm small holes, and appear on the lower surface of the membrane. The cells were cultured in this matrix gel chamber at 37˚C for 24 h in a CO_2 incubator and were fixed with 100% methanol and Giemsa stained. The cells on the upper portion of the matrix membrane were wiped off with cotton tips and cells on the other side of this membrane were observed by a phase-contrast microscopy. Number of invaded cells was counted in randomly selected 5 observation fields in a membrane at 100-fold magnification.

Treatment of HCC cell lines with IFN-α and sodium butyrate. Cancer cells were cultured in culture disks with natural IFN-α (Sumiferon®, Sumitomo Pharmaceutical Co., Osaka, Japan) at $1x10^2$ IU/ml and $1x10^3$ IU/ml or 2 mM of sodium butyrate (Sigma-Aldrich Japan) for 7 days. The medium was changed every 2 days. We selected the dose of sodium butyrate according to our preliminary experiments, which showed that 2 mM concentration of sodium butyrate induced HCC cells to significant reduction of their proliferation without inducing apoptosis. In some cell lines, increase in albumin production and reduction of α-fetoprotein production was induced in this concentration.

Gelatin zymography. HLE and HLF cells were cultured as described above with and without IFN or sodium butyrate for 7 days until the culture disk became confluent. The cells were recovered and washed 3 times with PBS and further cultured in serum-free medium for 48 h, and then the culture supernatant was obtained. The culture supernatant was concentrated with Centricon-10 (Millipore Co., Billerica,

MA) to 10-fold concentration, and this concentrated medium was used as the conditioned medium. The protein concentration in each medium was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Gelatinase activity in the conditioned medium was assessed by gelatin zymography following the methods described elsewhere (33). The conditioned medium which contained equal amounts of protein (10 μg) and the same volume of sample buffer (0.3 M Tris-HCl pH 6.8, 40% glycerol, 2% (w/v) SDS, 0.1% bromophenol blue) was mixed and incubated at 37°C for 30 min. The sample was electrophoresed on 9% SDS polyacrylamid gel containing 1% gelatin at 4°C at 30 mA. The gel was washed with 2.5% Triton X-100 twice and was incubated at 37°C for 15-18 h in TNC (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$) and was stained with Coomassie brilliant blue-R, followed by destaining with 5% isopropanol and 8% acetic acid.

Matrix metalloproteinase -2, and -9 activity assay. To investigate the mechanism of matrigel invasion and the effect of IFN and sodium butyrate, active form of MMP-2 and 9 was measured by the matrix metalloproteinase-2, and -9 activity assay system (34) (Amersham Bioscience Corp., Piscataway, NJ). The culture supernatant was obtained according to the procedure described above and was diluted 10-fold. This conditioned supernatant was applied to the measurement system. The procedure was achieved according to the instructions supplied by the manufacturer. The assay uses the pro-form of a detection enzyme that can be activated by capturing active MMP-2 or -9 and changes into an active detection enzyme. The MMP-activated detection enzyme was quantitatively measured using a specific chromogenic peptide substrate. The resultant color was read at 405 nm in a microtiter plate spectrophotometer. The concentration of active MMP-2 or -9 in the conditioned medium was determined by interpolation from a standard curve.

Effect of IFN and butyrate on the expression of MMP-1 and TIMP-1, -2. RNA was extracted from cancer cells with a modified acid guanidinium thiocyanate-phenol-chloroform method using Isogen (Nippon gene Co., Tokyo, Japan).

cDNA was synthesized from 5 μg of mRNA using ProSTAR™ First-Strand RT-PCR Kit (Stratagene, La Jolla, CA). The procedure was achieved according to the instructions supplied by the manufacturer. cDNA was labeled with SYBR Green during PCR (1 μl cDNA, 2X SYBR Green PCR Master Mix, each 5 pmol/μl of forward and backward primers; total 50 μl). PCR was performed and the products were detected using ABI PRISM 7700 detection system (Applied Biosystem, Foster City, CA). Reaction started at 50°C for 2 min and at 95°C for 10 min, and then 95°C 15 sec and 60°C 1 min for 40 cycles. A human fibrosarcoma-derived cell line HT-1080 was used as a calibration sample, and expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The primers used in this study were as follows: MMP-1 (35): forward, 5-CGG-TTT-TTC-AAA-GGG-AAT-AAG-TAC-T-3 (1092-2016); reverse, 5-TCA-GAA-AGA-GCA-GCA-TCGA TATG-3 (1219-1197); TIMP-1 (36): forward, 5-TGC-CGC-ATC-GCC-GAG-AT-3 (14-30); reverse, 5-ATG-GTG-GGT-TCT-CTG-GTG-3 (64-47); TIMP-2 (36): forward, 5-CAC-CAG-GCC-AAG-TTC-TTC-3 (858- 875); reverse, 5-CGG-TAC-CAC-GCA-CAG-GA-3 (916-900); GAPDH: forward, 5-GAA-GGT-GAA-GGT-CGG-AGT-C-3; reverse, 5-GAA-GAT-GGT-GAT-GGG-ATT-TC-3.

Statistical analysis. The data were expressed as mean ± SD. Statistical analysis was performed using Mann-Whitney's U test and $p<0.05$ was estimated to be significant.

Results

Morphological change of HLE and HLF treated with natural IFN-α and sodium butyrate. As the result of treatment with natural IFN-α and sodium butyrate, the shape and size of HLE and HLF cells were changed. With treatment of IFN-α 1000 U/ml for 7 days, the size of the cells became slightly larger and round-shaped, although hardly changed at 100 U/ml of IFN-α. On the other hand, with treatment of 2 mM sodium butyrate, the size of the cells became larger than that stimulated by IFN-α, especially nuclear size of each cell became larger (Fig. 1). The shape of HLF changed similarly with those of HLE. Apoptosis seemed not to occur in these cells during 7-day culture morphologically and TUNEL assay confirmed that apoptosis was not detected in this culture condition.

Mobility of the HCC cells. The mobility of HCC cells was quantitatively analyzed by digital image analyzer described in Materials and methods. In 2-day culture, HCC cells did not move enough in the observation field for evaluation of the effect of IFN-α and sodium butyrate by this system (data not shown). Therefore, invasion ability of HCC cells should be assayed by other procedures such as the Matrigel assay.

Effect of natural IFN-α and sodium butyrate on the Matrigel invasion activity of human HCC cells. Invasion activity through Matrigel of six human HCC cell lines was examined using Matrigel invasion assay. Invasion activity of HLE and HLF was high but that of other cell lines was very low (HLE, 160±38; HLF, 126±16; HCC-T, 4±2; HCC-M, 13±3; HepG2, 8±7; PLC/PRF/5, 7±6; the number indicated the number of cells invaded into Matrigel, went through small holes, and appeared on the other side of membrane) (Fig. 2). From this result, following experiments were performed using HLE and HLF. These two cell lines were treated with 100 IU/ml and 1000 IU/ml of IFN-α and 2 mM of sodium butyrate for 7 days, and the treated cells were applied on the Matrigel invasion assay. Although 100 IU/ml of IFN-α did not affect invasive ability of two human HCC cells, 1000 IU/ml of IFN-α and 2 mM of sodium butyrate significantly decreased invasive activity of these cell lines [HLE: control vs. IFN-treatment (1000 IU/ml), $p=0.0018$; control vs. SB-treatment, $p=0.0041$; HLF: control vs. IFN-treatment (1000 IU/ml), $p=0.0070$; control vs. SB-treatment, $p=0.0022$; Mann-Whitney's U test] (Fig. 3).

Effect of natural IFN-α and sodium butyrate on MMP-2 and -9 enzymic activities of human HCC cells. Gelatin zymography

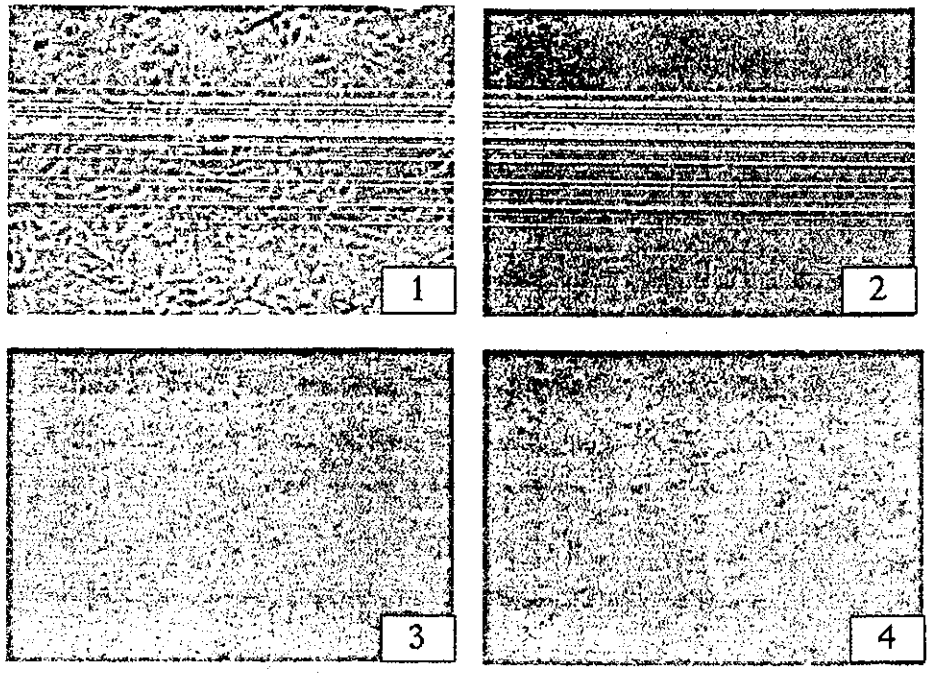

Figure 1. Morphologic change of HLE cells treated with natural IFN-α and sodium butyrate. As the result of treatment with natural IFN-α and sodium butyrate, the shape and size of HLE cells were changed. With the treatment of 1000 IU/ml of IFN-α for 7 days, the size of HLE cell became slightly larger and the morphology was round-shaped, although it hardly changed with 100 IU/ml of IFN-α. The treatment of HLE cells with 2 mM sodium butyrate changed the shape of cells more than that stimulated by IFN-α. Nucleic size of HLE cells became larger. 1, Control; 2, Cells treated with 100 IU/ml of IFN-α for 7 days; 3, Cells treated with 1000 IU/ml of IFN-α for 7 days; 4, Cells treated with 2 mM of sodium butyrate for 7 days. Original magnification x100.

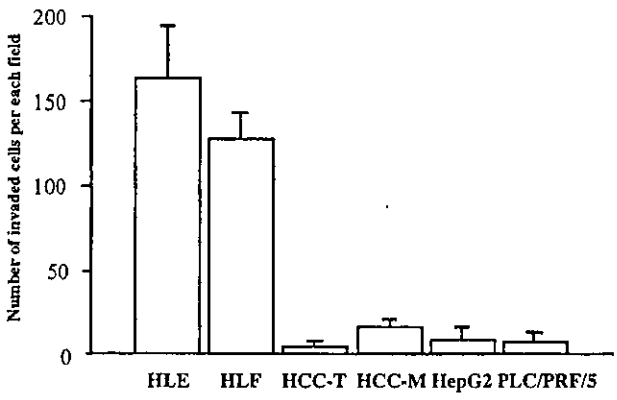

Figure 2. The morphometric analysis of the number of cells invaded from the upper-side chamber to the lower-side chamber in six human HCC cell lines. Invasion through Matrigel of HLE, HLF, HCC-T, HCC-M, HepG2 and PLC/PRF/5 was assayed using the Biocoat Matrigel Invasion Chamber. The cancer cells invade into the Matrigel, and appear on the lower surface of the Matrigel membrane. The number of invaded cells was counted in randomly selected 5 fields in every experiment (n=5).

showed that pro-MMP-2 and 9, and active MMP-2 and -9 were detected in the culture supernatants of both HLE and HLF. In HLE cells, production of MMP-2 was higher than that of MMP-9, and HLF produced much more MMP-9 than MMP-2. The culture supernatants of HLE and HLF treated with IFN-α (1000 IU/ml) and sodium butyrate (2 mM) for 7 days showed significant reduction of pro- and active-MMPs production in both cell lines. Especially MMP-2 activity almost disappeared by gelatin zymography in both cell lines (Fig. 4).

Effect of natural IFN-α and sodium butyrate on MMP-2 and -9 activities. Effect of agents on MMP activity was further examined using the MMP activity assay, which can detect active-form of MMP-2 and MMP-9. Active MMP-2 levels in HLE and HLF were 1.3±0.2 ng/ml and 0.5±0.2 ng/ml, respectively, in their control culture conditions. Active MMP-9 levels in HLE and HLF were 0.7±0.3 ng/ml and 1.2±0.3 ng/ml, respectively, in their control culture conditions. These levels almost disappeared by treatment with both IFN-α and sodium butyrate (Fig. 5).

Real-time quantitative RT-PCR assay. Effect of IFN-α and sodium butyrate on transcription of the MMP family and its counterpart, TIMP-1 and -2 was examined by real-time RT-PCR. The production of MMP-1 that dissolves type I collagen and promotes cancer cell invasion into its surrounding tissue was more or less inhibited by both agents. The mRNA levels of MMP-1 were slightly decreased by stimulation with 1000 IU/ml of IFN-α, and were significantly reduced by treatment with 2 mM of sodium butyrate. On the other hand, the agents variously changed the production of TIMP that inhibits MMP activity. The mRNA levels of TIMP-1 and TIMP-2, which are counterpart enzymes of MMP, were significantly increased by IFN-α ($p<0.01$, Mann-Whitney's U test) and slightly decreased by sodium butyrate (Fig. 6).

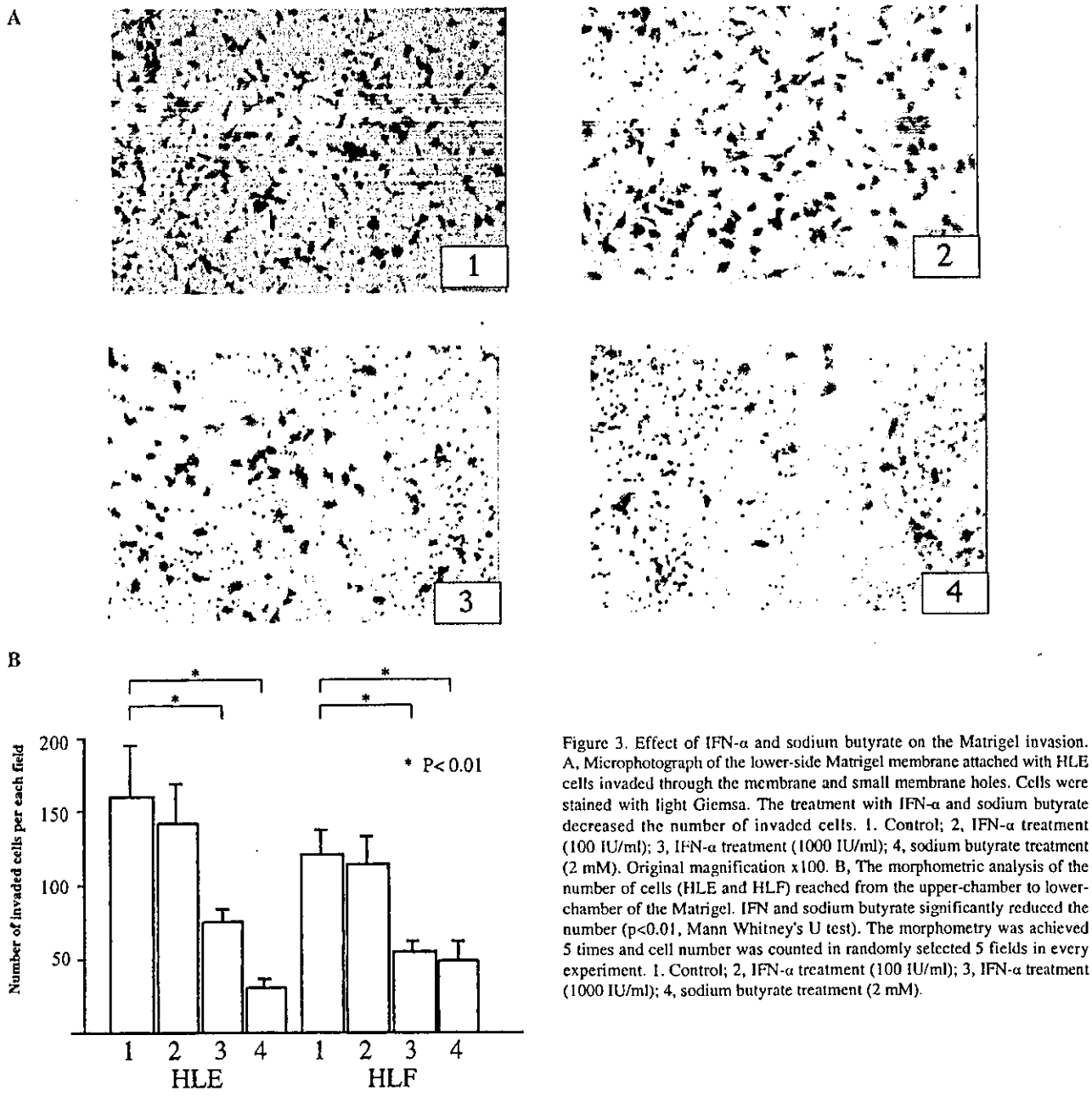

Figure 3. Effect of IFN-α and sodium butyrate on the Matrigel invasion. A, Microphotograph of the lower-side Matrigel membrane attached with HLE cells invaded through the membrane and small membrane holes. Cells were stained with light Giemsa. The treatment with IFN-α and sodium butyrate decreased the number of invaded cells. 1. Control; 2, IFN-α treatment (100 IU/ml); 3, IFN-α treatment (1000 IU/ml); 4, sodium butyrate treatment (2 mM). Original magnification x100. B, The morphometric analysis of the number of cells (HLE and HLF) reached from the upper-chamber to lower-chamber of the Matrigel. IFN and sodium butyrate significantly reduced the number ($p<0.01$, Mann Whitney's U test). The morphometry was achieved 5 times and cell number was counted in randomly selected 5 fields in every experiment. 1. Control; 2, IFN-α treatment (100 IU/ml); 3, IFN-α treatment (1000 IU/ml); 4, sodium butyrate treatment (2 mM).

Discussion

This study demonstrated that both IFN-α and sodium butyrate are potent inhibitors of malignancy of human HCC, especially of cellular invasive activity. Type I IFN has various anti-cancer activities, such as anti-proliferative activity, immuno-modulatory activity, and anti-angiogenic activity (37), in addition to anti-viral activity, and it has been clinically used in patients with renal cell carcinoma, multiple myeloma, chronic myelocytic leukemia, and hairy cell leukemia. Anti-malignant effect of IFN has been also reported in HCC. IFN induces apoptosis of human HCC cells (16), in case HCC cells are not resistant to apoptotic stimulation. Anti-apoptotic Bcl-2 family proteins may play an important role in this resistance (17,31,38,39). In this study, apoptosis was not induced and various aspects induced by IFN-α and sodium butyrate were confirmed not to be affected by apoptosis. *In vitro* studies demonstrated its anti-proliferative activity by inducing apoptosis or cell-cycle S-phase arrest. This cell-cycle arrest is considered to be due to p21/WAF1 induction and inhibition of cyclin A and B, which sequentially reduces cdk2 and cdc2 activities (30,40,41). Type I IFN was also demonstrated to inhibit angiogenesis of tumor vessels in nude mouse models (42,43). In addition, our previous studies demonstrated that IFN has an anti-metastasis effect by increasing cell-cell adhesion formed by E-cadherin and ß-catenin (25). Furthermore, telomerase activity was reduced and the present study demonstrated that IFN inhibits cancer cell invasion and metastasis by reducing MMP production of HCC cells. These observations confirm the

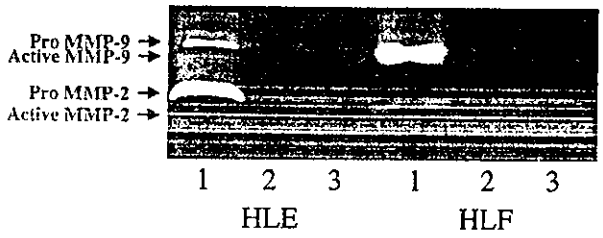

Figure 4. Gelatin zymography of the culture supernatants of HLE and HLF cells and the effect of IFN- and sodium butyrate-treatment. The serum-free culture supernatants of HLE and HLF with and without IFN and sodium butyrate for 7 days were collected. Equal amounts of protein were subjected to gelatin zymography described in Materials and methods. Destained band showed the existence of gelatinase. Control (lane 1), treated with 1000 IU/ml IFN-α (lane 2), treated with 2 mM SB (lane 3). Both HLE and HLF produced pro- and active MMP-2, and MMP-9. HLE produced MMP-2 much more than MMP-9, although HLF produced much more MMP-9 than MMP-2. The culture supernatants of HLE and HLF treated with IFN-α and sodium butyrate showed significant reduction of pro- and active-MMPs production in both cell lines. Especially MMP-2 activity almost disappeared in both cell lines. 1, Control; 2, IFN-α treatment (1000 IU/ml); 4, sodium butyrate treatment (2 mM).

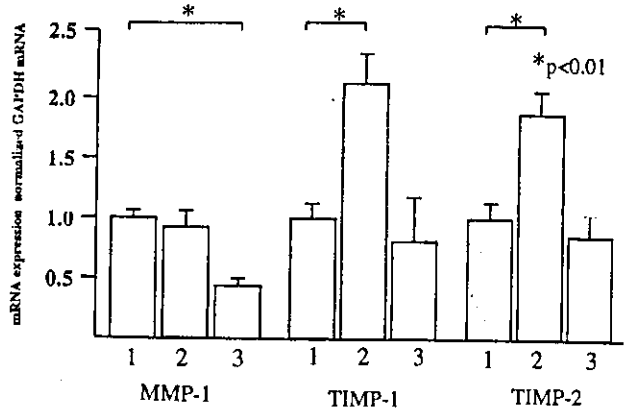

Figure 6. Real-time quantitative RT-PCR assay of MMP-1, TIMP-1 and TIPM-2. Effect of IFN-α and sodium butyrate on transcription of the MMP family and its counterpart, TIMP-1 and 2 of the HLE cell line was examined by real-time RT-PCR. The mRNA levels of MMP-1 decreased significantly by sodium butyrate. TIMP-1 and TIMP-2, which were counterparts of MMP-2, were significantly increased with treatment of 1000 U/ml of IFN-α ($p<0.01$, Mann Whitney's U test), although slightly decreased with treatment of 2 mM of sodium butyrate. Control (1), treated with 1000 IU/ml of IFN-α (2), treated with 2 mM of sodium butyrate (3).

theoretical background of recent clinical trials, which demonstrated that type I IFN was effective in advanced HCC (11,44) and inhibited secondary HCC raised after first treatment (45) other than acyclic retinoid (46,47). Recent studies have demonstrated that type I IFN directly activate p53 pathway not only in anti-viral but also in tumor suppression systems (48). We have demonstrated that interferon regulatory factor (IRF)-1 is a key transcription factor in the anti-proliferative activity of type I IFN (30), and it is possible that signal transduction of IFN depends on a type of single nucleotide polymorphisms in the IRF-1 promoter (18). Therefore anti-cancer effect of type I IFN on HCC may vary between individuals.

On the other hand, sodium butyrate is an HDAC inhibitor, which modifies histone acetylation and regulates gene expression (39). Recent studies revealed that a role of epigenetic conditions such as DNA methylation status and histone acetylation or methylation status are important factors in multistep carcinogenesis of human liver cancer especially that caused by chronic HCV infection. Hypomethylation in CpG islands of promoter regions in several oncogenes and deregulation of methylation enzymes have been demonstrated in human HCC (20,49,50). These epigenetic regulations of genomic expression are indispensable in human ontogeny or development and differentiation, suggesting that carcinogenesis and differentiation are closely related to each other. Sodium butyrate is categorized as one of differentiation inducers and its effects of increasing albumin production and decreasing α-fetoprotein production on HCC cells might be related to the cellular differentiation process.

MMP activity is considered to play an important role in invasion and metastasis activity of HCC. MMP-2 seems to be

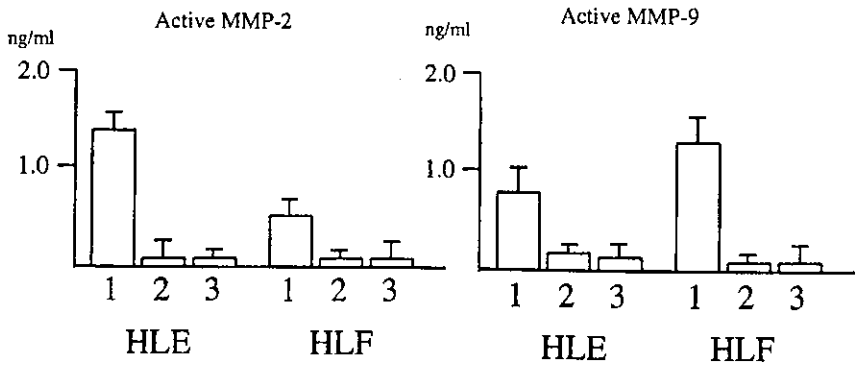

Figure 5. Quantitative analysis of active-form MMP-2 and -9 before and after treatment with IFN-α and sodium butyrate. The levels of active-type MMP-2 and -9 were assayed using the matrix metalloproteinase-2, -9 activity assay system. Control (1), treated with 1000 IU/ml of IFN-α (2), treated with 2 mM of sodium butyrate (3). Both active MMP-2 and MMP-9 levels produced by HLE cells were significantly decreased by IFN-α and sodium buytrate.

expressed in HCC especially in its peripheral region rather than central region of HCC, and the location is similar with that of MT1-MMP, which is an activator of MMP-2 (51), although negative data also exist (52). It seems true that the expression of active-form of MMP-2 is stronger in cancer than in non-cancer tissue in HCC (53,54). Active-forms of MMP-2 and -9 correlated with portal invasion, intrahepatic metastasis and recurrence rate after hepatic resection. MT1-MMP correlated with MMP-2 activation and invasion of HCC, and mRNA expression of this factor was positively shown in 22 cases in 30 HCCs. Ogata *et al* (54) demonstrated by immunostaining and *in situ* hybridization that expression of MT1-MMP and MMP-2 correlated to differentiation status of HCC tissues. Maatta *et al* (55) showed that MT1-MMP mRNA was expressed in HCC cells and its levels correlated to differentiation status of HCC, that is, the levels of expression is much more in poorly differentiated HCC than in well-differentiated type. MMP-2 expression levels of HCC may not be so different between cancer and the surrounding non-cancer tissue, but MMP-2 may be activated by MT1-MMP, which exists near the surrounding membrane of the tumor or membranous capsule, resulting in an increase of invasion potential (26). In addition, the balance of MMP-2 and TIMP-2 relates to metastasis potential (56). On the other hand, the localization of MMP-9 is closely correlated to tumor and malignant potential. Arii *et al* (52) showed that MMP-9 mRNA is detected only in the tumor, and its expression is higher in HCC with invasion through the membranous capsule. Immunostaining showed higher expression of this enzyme in the interface between tumor and non-tumor tissue. In the present study, both MMP-2 and MMP-9 expression and its activity was significantly reduced by IFN-α and sodium butyrate, suggesting that these two drugs are possibly useful for preventing HCC cells from invasion and metastasis.

Effect of IFN on MMP production or expression has been demonstrated in several types of cells. Type I IFN reduces MMP-2 production from glioma cells (57) and also reduces malignant potential of bladder cell carcinoma (58). These inhibitory effects of type I IFN on MMP production are induced via Stat-1 pathway in various types of cells (59,60). Another important mechanism may be a competitive binding of IRF-1 to NF-κB binding region in the MMP-9 promoter (61). It has been demonstrated that Stat-1, -2 and -3 are up-regulated by IFN-α in human HCC cell lines (62), and this pathway is responsible for up-regulation of MMP production.

In the present study, the effect of sodium butyrate, one of HDAC inhibitors, on HCC cells was similar with that of IFN-α, and our previous studies showed that effects of these two agents are very similar. It was demonstrated that Stat-1 expression is up-regulated in human HCC cell lines by sodium butyrate (63). This report suggests a possible crosstalk mechanism between sodium butyrate-induced and type I IFN-induced pathways. We investigated the gene expression after butyrate-stimulation in human HCC cells with a DNA micro-array, and one of the genes that increased by the stimulation was IRF-1, which is a key factor in the interferon system. This result also suggests that the reason why sodium butyrate shows a similar effect on HCC cells with IFN-α seems to be partially because of a crosstalk pathway between the effector systems in both agents. Other signal pathways may exist, because effects of these two agents on the expression of TIMP-1 and -2 were different from each other. Further investigation in these pathways may facilitate the development of a new therapeutic strategy.

Acknowlegments

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Herbal cardiotonic pills prevent gut ischemia/reperfusion-induced hepatic microvascular dysfunction in rats fed ethanol chronically

Yoshinori Horie, Jing-Yan Han, Shuka Mori, Masahiro Konishi, Mikio Kajihara, Takehiko Kaneko, Yoshiyuki Yamagishi, Shinzo Kato, Hiromasa Ishii, Toshifumi Hibi

Yoshinori Horie, Jing-Yan Han, Shuka Mori, Masahiro Konishi, Mikio Kajihara, Takehiko Kaneko, Yoshiyuki Yamagishi, Shinzo Kato, Hiromasa Ishii, Toshifumi Hibi. Department of Internal Medicine, School of Medicine, Keio University, Tokyo Japan
Supported by Grants From Tasley, Tianjin, China
Co-correspondents: Jing-Yan Han
Correspondence to: Yoshinori Horie, M.D., or Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan. yhorie@sc.itc.keio.ac.jp
Telephone: +81-3-3353-1211-62298 **Fax:** +81-3-3353-6247
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Abstract

AIM: Cardiotonic Pill (CP), an oral herbal medicine that includes Danshen (Salviae Miltiorrhizae), Panax notoginseny and Dyroblanops aromatica gaertn, has been clinically used for vascular diseases such as occlusive vasculitis, coronary diseases, atherosclerosis, and cerebral infarction. The main component, Salviae Miltiorrhizae, has been reported to prevent cerebral and intestinal reperfusion injury. However, little is known about the effect of CP on hepatic microcirculation. Thus, this study aimed to determine whether CP could affect hepatic microvascular dysfunction elicited by gut ischemia/reperfusion (I/R) in rats fed ethanol chronically.

METHODS: Male Wistar rats were pair-fed with a liquid diet containing ethanol or isocaloric control diet for 6 wk. After laparotomy, one lobe of the liver was examined through an inverted intravital microscope. The rats were exposed to 30 min of gut ischemia followed by 60 min of reperfusion. Rhodamine-6G-labeled leukocytes in the sinusoids were observed 90 min after the onset of superior mesenteric artery occlusion. Plasma tumor necrosis factor (TNF)-α and endotoxin levels were measured 1 h after the onset of reperfusion. Plasma alanine aminotransferase (ALT) activities were measured 6 h after the onset of reperfusion. In another set of experiments, CP (0.8 g/kg, intragastrically) was administered 1 and 24 h before the onset of ischemia.

RESULTS: In control rats, gut I/R elicited increases in the number of stationary leukocytes, and plasma TNF-α and endotoxin levels and plasma ALT activities. These changes were mitigated by pretreatment with CP. In ethanol-fed rats, the gut I/R-induced increases in the number of stationary leukocytes, plasma endotoxin levels and ALT activities were enhanced. Pretreatment with CP attenuated the enhancement of gut I/R-induced responses by chronic ethanol consumption.

CONCLUSION: These results suggest that CP prevents the gut I/R-induced hepatic microvascular dysfunction and hepatocellular injury. A reduction of inflammatory responses such as TNF-α production via reduction of blood endotoxin levels appears to be involved in the mechanisms. Chronic ethanol consumption enhances gut I/R-induced hepatic microvascular and hepatocellular injury. CP also attenuates an enhancement of gut I/R-induced responses by chronic ethanol consumption via the reduction of blood endotoxin levels.

Key words: Intestinal reperfusion injury; Hepatic microvascular dysfunction; Cardiotonic Pill; Ethanol

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INTRODUCTION

A large body of evidence implicates leukocytes as mediators of microvascular dysfunction and tissue injury associated with reperfusion of ischemic organs. Several experimental strategies have been used to demonstrate the contribution of leukocytes to ischemia/reperfusion (I/R) injury, including polyclonal antibodies that render animal leukopenia[1-3], adhesion molecule-specific monoclonal antibodies[1,4-6], and adhesion molecule deficiency in mice[7,8]. The effectiveness of adhesion molecule-specific monoclonal antibodies (MAbs) and adhesion molecule-deficiency in attenuating I/R-induced tissue injury have led to the widely held view that leukocyte-endothelial cell adhesion is a rate-determining step in the pathogenesis of this injury process. We developed a leukocyte-dependent model of hepatocellular dysfunction elicited by gut I/R[1,7,9]. This murine model allows *in vivo* assessment of the effects of I/R on leukocyte sequestration in sinusoids of different regions of the liver lobule, leukocyte adherence in postsinusoidal venules, and the number of perfused sinusoids.

Ethanol has also been reported to modulate I/R-induced tissue injury[10-12]. In a perfused liver model, ethanol enhanced I/R-induced hepatotoxicity (an increase in blood levels of liver enzymes) by an enhanced production of reactive oxygen species[10]. In an *in vivo* gut I/R model, ethanol also enhanced gut I/R-induced neutrophil accumulation in the intestinal wall[11]. In an *in vivo* cerebral I/R model, however, ethanol pretreatment was reported to reduce cerebral I/R injury[12]. We have recently reported that low-dose ethanol attenuates gut I/R-induced hepatic microvascular dysfunction in the midzonal region and sequential hepatocellular injury, whereas high-dose ethanol enhances hepatic microcirculatory disturbances in the pericentral region and sequential hepatocellular injury[13].

Clinically, long-term alcohol consumption has been noted to significantly reduce the incidence of coronary artery disease[14]. However, chronic alcohol consumption often results in fatty liver and liver failure, which is of particular concern when such fat-laden tissues are used as donor organs in liver transplantation. This important clinical problem has drawn attention to the relationship between ethanol consumption and reperfusion

injury in the liver. Both gut I/R and chronic consumption of ethanol are known to cause liver injury via mechanisms that involve oxidative stress and microcirculatory disturbances that include leukocyte sequestration and sinusoidal malperfusion[15]. Gut I/R is known to produce an elevation in plasma endotoxin levels (9), while chronic ethanol consumption has been reported to enhance the hepatic microcirculatory dysfunction and hepatocellular injury induced by endotoxins[16-18]. Based on these observations one might expect that chronic ethanol consumption would lead to an exaggerated liver injury response to gut I/R. Furthermore, we have recently reported that chronic ethanol consumption enhances hepatic leukosequestration, impaired sinusoidal perfusion, and hepatocellular injury caused by gut I/R via expression of intercellular adhesion molecule (ICAM)-1[19].

Herbal medicines (traditional medicines from natural sources) are recently attracting increased global attention. Further, Chinese herbal medicines have stood the test of time and many are widely accepted as having reliable therapeutic efficacy. Cardiotonic Pill (CP), an oral herbal medicine that includes Danshen (Salviae Miltiorrhizae), Panax notoginseny and Dyroblanops aromatica gaertn, has been clinically used in China, Korea and Russia for vascular diseases such as occlusive vasculitis, coronary diseases, atherosclerosis, and cerebral infarction, which are related to microvasclar dysfunction. In clinical cases, the main component, Danshen, has been reported to have curative efficacy in ischemic cerebrovascular disease. In animal models, Danshen has been reported to prevent cerebral and intestinal reperfusion injury[20,21]. In the rat cerebral ischemia model, Danshen was reported to improve cerebral blood flow in the ischemic hemisphere and to inhibit platelet aggregation in rats[20]. However, little is known about the effect of CP on hepatic microcirculation. Hence, the overall objectives of this study was to assess the effects of CP on hepatic microvascular dysfunction and hepatocellular injury induced by gut I/R, and to determine whether CP could affect the hepatic microvascular dysfunction elicited by gut I/R in rats fed ethanol chronically.

MATERIALS AND METHODS

Animals

Thirty (15 pairs) male Wistar rats weighing about 150 g were pair-fed for 6 wk with a liquid diet containing ethanol that provided 36% of the total dietary calories or an isocaloric control diet according to the method of Lieber *et al.*[22]. All rats were fasted for18 h prior to the experiments, which were all performed according to the criteria outlined in the US National Research Council and the Keio Animal Research Guides.

Intravital microscopy

Rats were anesthetized with pentobarbital sodium (35 mg/kg) intraperitoneally. The left carotid artery was cannulated and a catheter placed at the aortic arch to monitor blood pressure. The left jugular vein was also cannulated for drug administration. After laparotomy, a lobe of the liver was observed with an inverted intravital microscope (TMD-2S, Diaphoto, Nikon, Tokyo, Japan) assisted by a silicon intensified target (SIT) camera (C-2400-08, Hamamatsu Photonicus, Shizuoka, Japan). The liver was placed on an adjustable Plexiglas microscope stage with a non-fluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The liver was carefully placed to minimize the influence of respiratory movements, and the surface was moistened and covered with cotton gauze soaked with saline. Images of the microcirculation were observed from the surface of the liver through a ×20 fluorescent objective, and microfluorographs were recorded on videotape using a videocassette recorder (S VHS-HQ, Victor, Japan).

Analysis of leukocyte accumulation

Leukocytes were labeled *in vivo* with rhodamine-6G (1 mg was dissolved in 5 mL of 0.9% saline) using a previously described method[12,17,19,23], which was based on a method used in rat brain[3]. It was shown that rhodamine 6G could selectively stain white blood cells and platelets, but not endothelial cells[3]. Thus, the fluorochrome allowed for differentiation between adherent leukocytes and endothelial cells. Rhodamine-6G (0.2 mL/100 g body weight) was injected prior to ethanol administration with subsequent injections every 30 min. Rhodamine-6G associated fluorescence was visualized by epi-illumination at 510-560 nm, using a 590 nm emission filter. We selected one of the lobules, which had well-perfused sinusoids and the fewest stationary leukocytes, choosing the furthest lobule from the edge of the liver if all the conditions were thought to be equivalent. A microfluorograph of hepatic microcirculation, with rhodamine-6G-labeled leukocytes in the sinusoids, was continuously observed for 90 min after the SMA occlusion and recorded on a digital video recorder for 1 min at 0, 30, 60, and 90 min. The number of stationary leukocytes was determined off-line during playback of the videotape images. A leukocyte was considered stationary within the microcirculation (sinusoids) if it remained stationary for more than 10 s. The lobule, which had the fewest stationary leukocytes was selected for observation at a basal condition. Stationary leukocytes were quantified in both the midzonal and pericentral regions of the liver lobule and expressed as the number per field of view (2.1×10^5 μm²).

Experimental protocols

We observed the surface of the liver for 10 min before ligation of the superior mesenteric artery in order to ensure that all parameters measured on-line were in a steady state. The superior mesenteric artery was then ligated with a snare created from polyethylene tubing for 0 (sham) or 30 min. After the ischemic period, the ligation was gently removed. Leukocyte accumulation was measured before ischemia, immediately following reperfusion and every 30 min for one hour thereafter.

In another set of experiments, CP (0.8 g/kg, intragastrically, Tasley, Tianjin, China) was administered at 1 and 24 h before the onset of ischemia to both control and ethanol-fed rats, and the same protocol was performed. Twenty-five mg (one pill) of CP includes 9 mg of Danshen (Salviae Miltiorrhizae), 1.76 mg of Panax notoginseny, 0.5 mg of Dyroblanops aromatica gaertn, and 13.74 mg of polyethylene glycol.

Liver enzyme, endotoxin and TNF assays

Sixty min after the onset of reperfusion, the rats were removed from the microscope stage and the abdominal wall was closed. Blood (plasma) samples for endotoxin and tumor necrosis factor (TNF)-α level measurement were collected from the inferior vena cava at a point proximal to the hepatic vein 1 h after the onset of reperfusion. For the measurement of endotoxin levels, blood samples were also collected from the portal vein. Samples for plasma alanine aminotransferase (ALT) measurement were obtained 6 h after the onset of reperfusion. Plasma ALT activity was determined by conventional UV method as previously described[24]. Plasma TNF-α concentration was determined in a microtiter plate using a TNF-α immunoassay kit (BioSource International, Camarillo, CA) based on enzyme-linked immuno sorbent assay (ELISA). In accord with our previous report[25,26], plasma endotoxin levels were measured by endospecy (an endotoxin-specific chromogeic limulus regent; Seikagaku Co., Tokyo, Japan) using an automated kinetic assay for endotoxin[27].

Statistical analysis

The data were analysed using standard statistical analyses, i.e.,

ANOVA and Scheffe's (post hoc) test. All values were reported as mean±SD, with five rats per group. Statistical significance was set at $P<0.05$.

RESULTS

Table 1 shows the effects of CP treatment on leukostasis in sinusoids of the midzonal and pericentral regions and the terminal hepatic venule (THV), plasma ALT activities, and plasma endotoxin and TNF-α levels. Pretreatment with CP *per se* did not affect any values of these in control rats. Chronic ethanol feeding *per se* caused leukostasis in the pericentral region of the liver, while CP diminished it.

Figure 1A illustrates the effects of CP treatment on gut I/R-induced leukostasis in sinusoids of the midzonal and pericentral regions and the THV (Panel A) of the liver lobule, and the entire liver lobule (sinusoids+THV, panel B) in the presence or absence of chronic ethanol consumption In control rats, gut I/R elicited increases in the number of stationary leukocytes in both hepatic sinusoids and THV. In ethanol-fed rats, the gut I/R-induced leukostasis was blunted in the midzonal region (control; 12.6±1.4, ethanol: 7.2±0.7, per field), while exaggerated leukostasis was noted in the pericentral region (control; 4.3±1.9, ethanol: 6.8±2.2) and THV (control; 4.0±1.4, ethanol: 13.0±1.6, per field). Although the leukostasis elicited by gut I/R in control rats was attenuated by the pretreatment with CP (leukostasis in the pericentral region; 4.0±1.2, THV; 1.8±0.7, per field), the exaggerated leukostasis in ethanol-fed rats was largely prevented by pretreatment with CP (leukostasis in the pericentral region; 3.0±0.7, THV; 4.8±1.2, per field).

Figure 1B shows the effects of CP treatment on plasma ALT activity following gut I/R in the presence or absence of chronic ethanol consumption. In control rats, gut I/R led to an elevation of plasma ALT activities. Chronic ethanol consumption enhanced the gut I/R-induced increase in plasma ALT activities (control; 146±31 IU/L, ethanol: 236±84 IU/L). The increase in plasma ALT activities elicited by gut I/R in both control and ethanol-fed rats was significantly attenuated by pretreatment with CP (control; 81±19 IU/L, ethanol: 108±58 IU/L).

Figure 1C shows the effects of CP treatment on plasma endotoxin levels following gut I/R in the presence or absence of chronic ethanol consumption. Gut I/R caused a slight elevation

Table 1 Effect of cardiotonic pills on leukostasis in liver, plasma ALT activity, and plasma endotoxin and TNF-α level in control and ethanol-fed rats

Group	Leukostasis midzonal (per field)	Pericentral (per field)	THV (per field)	ALT activity (IU/L)	Endotoxin (pg/mL)	TNF-α (pg/mL)
Control	1.8±0.7	1.0±0.7	0.5±0.5	31.6±12.0	9.5±5.3	17.0±8.9
Ethanol	1.4±0.5	3.2±0.5*	1.2±0.7	40.6±10.5	19.2±10.8	23.1±15.1
Control+CP	1.4±1.2	1.0±1.0	0.2±0.5	28.4±11.8	9.3±5.3	13.3±10.1
Ethanol+CP	1.6±0.5	0.8±0.5	0.6±1.0	36.6±7.2	13.6±5.5	10.6±6.2

CP: cardiotonic pills, *$P<0.05$ *vs* Control.

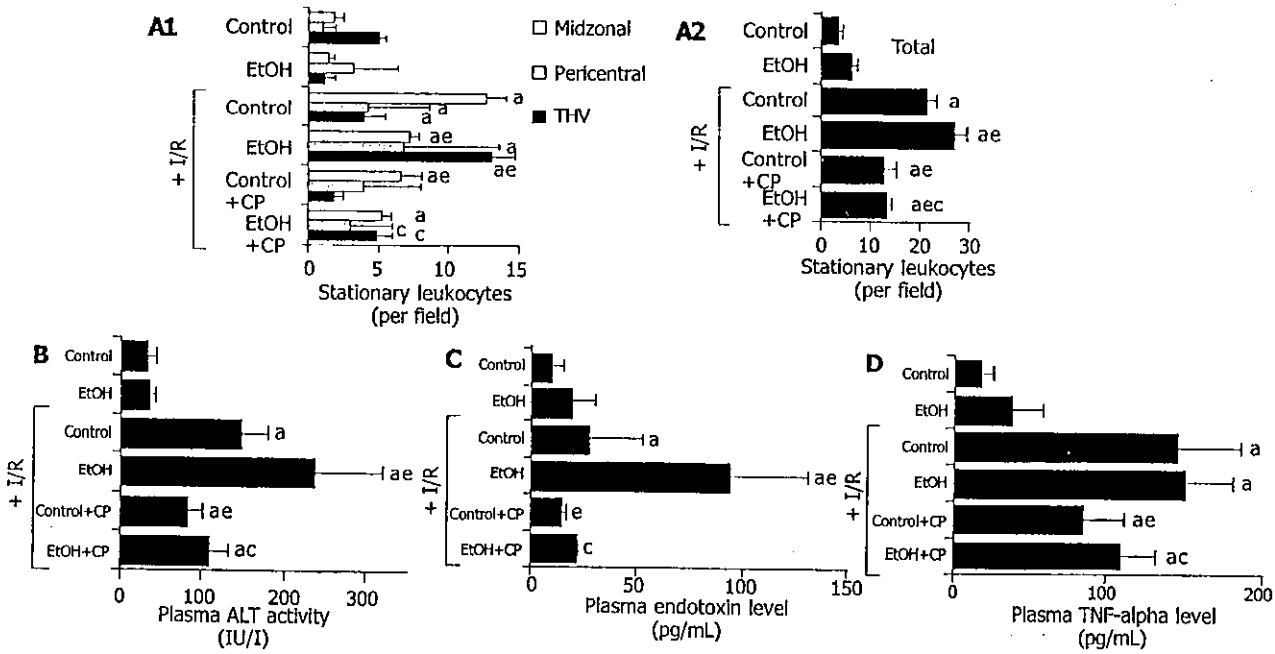

Figure 1 Effects of chronic ethanol consumption and/or Cardiotonic Pill (CP) on the number of stationary leukocytes, plasma ALT activities, plasma endotoxin concentration, and plasma TNF-α concentration after gut I/R. Each group consisted of five animals. *$P<0.05$ *vs* control, †$P<0.05$ *vs* Control+I/R, ‡$P<0.05$ *vs* ethanol+I/R. A: Effects of chronic ethanol consumption and/or CP on the number of stationary leukocytes in each region (the midzonal and pericentral regions) (panel A) and the entire (combined) liver lobule (panel B) after 30 min of gut ischemia and 60 min of reperfusion; B: Effects of chronic ethanol consumption and/or CP on plasma ALT activities at 6 h after gut I/R; C: The effects of chronic ethanol consumption and/or CP on plasma endotoxin concentration after 30 min of gut ischemia and 60 min of reperfusion; D: The effects of chronic ethanol consumption and/or CP on plasma TNF-α concentration after 30 min of gut ischemia and 60 min of reperfusion.

of plasma systemic and portal endotoxin levels in control rats, while chronic ethanol consumption enhanced the gut I/R-induced increase in plasma endotoxin levels (control; 26.2± 27.1 pg/mL, ethanol: 93.2±51.4 pg/mL). The exaggerated elevation of plasma endotoxin levels in ethanol-fed rats was largely prevented by the pretreatment with CP (endotoxin levels; 21.3± 1.4 pg/mL).

Figure 1D summarizes the effects of CP treatment on gut I/R-induced increase in plasma TNF-α levels in the presence or absence of chronic ethanol consumption. In control rats, gut I/R elicited a significant increase in plasma TNF-α levels. Although chronic ethanol consumption did not affect gut I/R-induced increases in plasma TNF-α levels, CP treatment reduced plasma TNF-α in both control and ethanol-fed rats.

DISCUSSION

Our previous study[19] has demonstrated that leukocyte-endothelial cell adhesion is an important determinant of the exaggerated microvascular dysfunction and tissue injury after gut I/R in the liver of rats fed ethanol chronically. The main component of CP is Danshen. Seven water-soluble components have been isolated from the Danshen root, Radix Salviae Miltiorrhizae. Using HPLC, at least 10 peaks were resolved based on its affinity to α 1-acid glycoprotein[28]. One of the components, salvianolic acid, was reported to protect cerebral I/R injury in rats[20]. Althogh CP had a protective effect on carbon tetrachloride-induced hepatocellular injury[29], the effect of CP on either hepatic I/R injury or hepatic microcirculation has not been reported. In this study, we demonstrated the protective effects of CP on gut I/R-induced liver injury in rats fed ethanol chronically.

Reperfusion of the ischemic intestine in control rats resulted in an accumulation of adherent leukocytes in sinusoids and THV, a reduction in the number of perfused sinusoids, and the release of liver enzyme (ALT) into the blood stream. In control rats, gut I/R-induced leukostasis in the pericentral region and THV were not noted after pretreatment with CP. This pretreatment also attenuated the gut I/R-induced increase in plasma ALT and TNF-α levels. An interesting finding in the present study is that the gut I/R-induced increase in plasma endotoxin level was not seen in control rats after the pretreatment with CP. Gut I/R was reported to elevate plasma endotoxin levels, which appeared to be derived from the gut[19]. CP has been reported to blunt mesenteric I/R injury[21]. Endotoxin has been known to cause hepatic microvascular dysfunction and hepatocellular injury[17,18,24]. Taken together, these results and evidence from the literature suggest that CP can reduce blood endotoxin levels by protecting the intestinal mucosal barrier from I/R injury, thereby preventing the subsequent hepatic microvascular dysfunction and hepatocellular injury.

Chronic ethanol consumption exaggerated gut I/R-induced leukostasis in the liver, gut I/R-induced increase in plasma endotoxin levels, and the subsequent hepatocellular injury (ALT elevation). The findings in the present study lend support to the possibility that elevated plasma levels of endotoxin contribute to the exaggerated inflammatory and tissue injury responses seen in the liver after gut I/R in rats chronically fed ethanol. The portal endotoxin level in rats fed ethanol chronically was higher than that in controls[19]. This result suggested that intestinal mucosal permeability increased in ethanol-fed rats after gut I/R. However, systemic endotoxin levels were much lower than portal endotoxin levels in control rats, whereas there was no significant difference between systemic and portal endotoxin levels in ethanol-fed rats after gut I/R[19], suggesting that clearance of endotoxin was impaired in ethanol-fed rats. Thus, both an increase in intestinal mucosal permeability and impaired clearance of endotoxin in ethanol-fed rats could be involved in the enhancement of plasma endotoxin levels. In the present study, pretreatment with CP substantially reduced the exaggerated increase in plasma endotoxin levels in ethanol-fed rats to almost the same level as that in untreated rats (without I/R) This result suggests that CP might blunt the enhanced increase in intestinal mucosal permeability and /or improvesed the impaired clearance of endotoxin in ethanol-fed rats.

Although chronic ethanol consumption enhanced the gut I/R-induced increase in plasma endotoxin levels, it did not affect the gut I/R-induced increase in plasma TNF-α levels. Chronic ethanol consumption enhanced the gut I/R-induced increase in plasma ALT activities with a parallel increase in leukostasis in the liver. These results suggest that leukostasis *per se* or leukocyte-derived oxidants may play a more important role in gut I/R-induced liver (hepatocellular) injury than cytokines. Another likely interpretation is that cytokines other than TNF-α are involved in the enhanced responses after gut I/R in rats fed ethanol chronically. In the present study, however, CP decreased the gut I/R-induced increase in plasma ALT activities with a parallel decrease in plasma TNF-α levels. Moreover, CP also attenuated the enhancement of gut I/R-induced increase in plasma ALT activities in ethanol-fed rats with a parallel attenuation of plasma TNF-α levels. These results indicate that CP can reduce the production of TNF-α independent of endotoxin levels. CP was also reported to blunt TNF-α-induced endothelial cell injury via NF-κB activation[30]. Therefore, CP may prevent gut I/R-induced hepatic microvascular dysfunction via protection of endothelial cells.

Thus, CP can protect gut I/R-induced hepatic microvascular dysfunction and hepatocellular injury. Although further studies and clinical trials are required, CP appears to have therapeutic usefulness against reperfusion injury in the liver.

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意識障害

加藤章信* 岩井正勝*
佐原圭* 鈴木一幸*

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1 はじめに

意識障害や昏睡の患者に対応する場合，患者自身から病歴が得られることは少なく，病歴聴取が重要なポイントとなる．また，意識レベルを的確に判断して，意識レベルが改善しているのか，悪化しているのか速やかに判断することも必要となる．意識障害の原因鑑別とともに初期治療も始めなければならないことから，さまざまな疾患を念頭におきながら鑑別が必要となる．意識障害と，肝胆膵疾患とのかかわりでは肝性脳症が上げられる．

肝性脳症は重篤な肝障害あるいは門脈大循環短絡に起因する精神神経症状であり，軽症のものから深昏睡まで幅がある．また精神神経症状が明らかでなく定量的精神神経機能検査で初めて指摘される潜在性肝性脳症がある．診断は肝機能異常，精神神経症状，高アンモニア血症，脳波などから総合的になされる．治療に抵抗性の肝性脳症の場合には早期に専門医への搬送が必要となる．

2 意識障害の病態

「意識がある」とは自己と外界との関連を正しく認識している状態で，周囲に対して適切な対応ができる状態である．「意識がある」あるいは「覚醒している」状態に関与する部位は脳幹部の上行性網様体賦活系と視床下部ならびに視床を介して大脳皮質に広汎に投射される経路がある．これらの経路のどのレベルで障害されても意識障害は生ずることとなる．

脳出血や脳梗塞といった頭蓋内病変により意識障害（一次的脳障害）は生じるが，脳幹網様体から大脳皮質の機能を広範に低下させる病態でも意識障害が生じる．すなわち，ショックによる低血圧，一酸化炭素中毒などによる酸素利用率の低下，低血糖などによるグルコース利用率の低下，さらに臓器不全による中間代謝物の蓄積（アンモニアなど）といった脳の機能を低下させる外因性の誘因によっても意識障害が生じ，このような病態を二次性代謝性脳症（昏睡）といい，肝性脳症

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*岩手医科大学第1内科学教室［〒020-8505 盛岡市内丸19-1］

表1　急性期意識障害のJapan coma scale（JCS）による分類（3-3-9度方式）

Ⅰ．刺激しないでも覚醒している状態（1桁で表現）
（delirium，confusion，senselessness）
1．だいたい意識清明だがいま一つはっきりしない
2．見当識障害がある
3．自分の名前，生年月日がいえない
Ⅱ．刺激すると覚醒する状態-刺激をやめると眠り込む-（2桁で表現）
（stupor，lethargy，hypersomnia，somnolence，drowsiness）
10．普通の呼びかけで容易に開眼する
合目的的な運動（たとえば，右手を握れ，離せ）をするし，言葉も出るが，間違いが多い*
20．大きな声または体を揺さぶることにより開眼する
簡単な命令に応ずる．たとえば握手*
30．痛み刺激を加えつつ呼びかけを繰り返すとかろうじて開眼する
Ⅲ．刺激をしても覚醒しない状態（3桁で表現）
（deep coma，coma，semicoma）
100．痛み刺激に対し，払い除けるような動作をする
200．痛み刺激で少し手足を動かしたり，顔をしかめる
300．痛み刺激に反応しない（deep coma，coma）

注　R：restlessness（不穏），I：incontinence（失禁），
A：akinetic mutism，apallic state（無言無動，自発性喪失）
例　10-I，20-RI，3-A
*何らかの理由で開眼できない場合

表2　Glasgow coma scale（GCS，1977）

		スコアー
A．開眼	自発的に（spontaneous）	E4
（eye opening）	言葉により（to speech）	3
	痛み刺激により（to pain）	2
	開眼しない（nil）	1
B．言葉による最良応答	見当識あり（orientated）	V5
（best verbal response）	錯乱状態（confused conversation）	4
	不適当な言葉（inappropriate words）	3
	理解できない言葉（incomprehensible sounds）	2
	なし（nil）	1
C．運動による最良反応	命令に従う（obeys）	M6
（best motor response）	痛み刺激部位に手を持ってくる（localizes）	5
	痛み刺激に逃避（withdraws）	4
	異常屈曲（abnormal flexion）	3
	疼痛で四肢伸展（extends）	2
	まったく動かさない（nil）	1

はこの範疇に含まれる．

3 意識障害の評価スケール

意識障害を評価するスケールにはさまざまな方法があるが，3-3-9度方式による分類（表1）とGlasgow coma scale（GCS）による分類（表2）が広く利用されている．ことに3-3-9度方式は簡便であり，看護師，救急隊員などの医療従事者にも利用できる．GCSは国際的に認知度が高いが，開眼，言語，運動の3つのパラメータを用いてその総和として表現するため煩雑である．15点満点で急性頭

表3　意識障害の原因疾患

一次性脳障害	脳血管障害	脳梗塞，脳出血，くも膜下出血など
	頭部外傷	脳挫傷，急性硬膜外血腫，急性硬膜下血腫 外傷性脳内血腫，びまん性軸索損傷，慢性硬膜下血腫
	脳腫瘍	髄膜腫，神経膠腫，転移性脳腫瘍など
	頭蓋内感染症	脳膿瘍，髄膜炎，脳炎
二次性脳障害	代謝性脳障害	低血糖，高血糖，肝性昏睡，尿毒症性昏睡，先天性代謝異常ビタミン欠乏（Wernicke脳症など），内分泌疾患（Addison病，粘液水腫など），電解質異常
	循環・酸素障害	ショック，重症心不全，一酸化炭素中毒，低酸素血症など
	薬物中毒	農薬中毒，向精神薬中毒，急性アルコール中毒
	その他	熱中症
その他		低体温，精神疾患，ヒステリー，てんかん

部外傷の場合7点以下は重症で予後が悪いと推定される．

肝性昏睡では犬山シンポジウムにより定められた肝性脳症の昏睡度分類を用いる．3-3-9度方式と肝性脳症の昏睡度分類の対応は表4に示すごとくである．

4 意識障害の原因

1）一次性脳障害（脳に限局性に病変を有する場合）：脳血管障害，頭部外傷，脳腫瘍頭蓋内感染症など

2）二次性脳障害：①代謝性脳障害（肝性脳症はこの範疇に入る）②ショックなどにより脳への血流や酸素供給が不足して生じるものなどがある．

3）その他：精神疾患やヒステリーなど（表3）

5 病歴聴取のポイント（図1）

意識障害があることから患者本人からの聴取は困難である．そのため家族や発見者から，発症時の状況と経過を詳しく聞き取る必要がある．同時に既往歴や服薬内容を調査することも重要である．病歴のみから原因を判断できることも多い．また，外傷による脳症のこともあることから身体所見のチェックも重要である．また，意識障害の前に激しい頭痛がなかったか，麻痺や発熱がなかったか，さらに睡眠薬や農薬などの容器がなかったか確認する必要もある．また，糖尿病例の場合にはインスリンの使用時間や食事時間の聴取も必要である．さらに内分泌疾患や肝臓，腎臓の既往は必ず聞く必要がある．しかし，意識障害の患者が搬入される場合に，付き添いもなく，病歴も明らかでないことがしばしばあり，この場合，重要な既往歴や経過を見逃す危険を常に意識して診察する必要がある．

肝性脳症の診断には，肝性脳症という病態があるということを思い起こすことも重要である．

1．初期鑑別診断のポイント

初期治療と平行して鑑別を進める．まず，意識障害に伴う舌根沈下に対して気道確保を行い必要に応じて酸素投与を行う．さらに血管確保を行うといったバイタルサインの異常に対する処置が優先される．

さらに採血し血液ガス分析のみならず，血液生化学検査を測定する．血糖値についてはデキスターチェックなどによりできるだけ迅速に測定することが必要である．