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A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route

Keiji Matsubayashi, Jong-Hon Kang, Hidekatsu Sakata, Kazuaki Takahashi, Motohiro Shindo, Masaru Kato, Shinichiro Sato, Toshiaki Kato, Hiroyuki Nishimori, Kunihiko Tsuji, Hiroyuki Maguchi, Jun-ichi Yoshida, Hiroshi Maekubo, Shunji Mishiro, and Hisami Ikeda

BACKGROUND: Five cases of transfusion transmission of hepatitis E virus (HEV) have been reported so far. The infection routes of the causative donors remain unclear, however. Also, the progress of virus markers in the entire course of HEV infection has not been well documented.

STUDY DESIGN AND METHODS: Nucleic acid testing was performed by real-time reverse transcription–polymerase chain reaction targeting the open reading frame 2 region of HEV. Full-length nucleotide sequences of HEV RNA were detected by direct sequencing.

RESULTS: Lookback study of a HEV-positive donor revealed that the platelets (PLTs) donated from him 2 weeks previously contained HEV RNA and were transfused to a patient. Thirteen relatives including the donor were ascertained to enjoy grilled pork meats together in a barbecue restaurant 23 days before the donation. Thereafter, his father died of fulminant hepatitis E and the other 6 members showed serum markers of HEV infection. In the recipient, HEV was detected in serum on Day 22 and reached the peak of 7.2 log copies per mL on Day 44 followed by the steep increase of alanine aminotransferase. Immunoglobulin G anti-HEV emerged on Day 67; subsequently, hepatitis was resolved. HEV RNA sequences from the donor and recipient were an identical, Japan-indigenous strain of genotype 4. HEV RNA was detectable up to Day 97 in serum, Day 85 in feces, and Day 71 in saliva.

CONCLUSION: A transfusion-transmitted hepatitis E case by blood from a donor infected via the zoonotic food-borne route and the progress of HEV markers in the entire course are demonstrated. Further studies are needed to clarify the epidemiology and the transfusion-related risks for HEV even in industrialized countries.

Hepatitis E virus (HEV) infection has been considered to occur mainly via fecal-oral transmission and is an important public health concern in developing countries.[1] In industrialized countries including Japan, cases have been rarely reported and hepatitis E has been regarded as an imported infectious disease from its endemic areas. Recently, however, increasing numbers of sporadic cases have been reported,[2-11] some of which resulted from infection via a zoonotic food-borne route by consumption of raw or undercooked meats of wild boar, wild deer, or farmed pig that was contaminated with HEV.[8-11]

In 2004, we reported the first molecularly confirmed case of transfusion transmission of HEV.[12] The infection route in the causative donor was not very clear, however. Thereafter, at least four cases of transfusion transmission of HEV have been reported in Japan, the United Kingdom,

ABBREVIATIONS: FAM = 6-carboxyfluorescein; HEV = hepatitis E virus; ORF = open reading frame; PSL = predonisolone; TAMRA = 6-carboxy-tetramethylrhodamine.

From the Hokkaido Red Cross Blood Center, Sapporo; the Center for Gastroenterology, Teine Keijinkai Hospital, Sapporo; the Department of Medical Sciences, Toshiba General Hospital, Tokyo; the Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa; the Department of Internal Medicine, Kitami Red Cross Hospital, Kitami; and the Department of Internal Medicine, Teine Keijinkai Hospital, Sapporo, Japan.

Address reprint requests to: Keiji Matsubayashi, Hokkaido Red Cross Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo 063-0002, Japan; e-mail: kmatsu@hokkaido.bc.jrc.or.jp.

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and France,[13-16] where hepatitis E is nonendemic and HEV infection routes remained to be obscure.

Here, we report a case of acute hepatitis E caused by transfusion transmission from the donor who was infected with HEV via a zoonotic food-borne manner. To our knowledge, this is the first case in which the infection route of the causative donor has been confirmed. Also, in this report, we describe, for the first time, the virus kinetics and changes of anti-HEV in serum, prospectively monitored from latent period of infection until convalescence, accompanied by disease progression in the patient.

MATERIALS AND METHODS

Detection and quantitation of HEV RNA

For reverse transcription–polymerase chain reaction (RT-PCR) to detect HEV RNA in the samples, the following oligonucleotides were designed to detect 75 nucleotides of highly conserved sequence in the open reading frame (ORF) 2 region of all HEV genotypes: forward primer 5′-CGGCGGTGGTTTCTGG-3′, reverse primer 5′-AAGG GGTTGGTTGGATGAATA-3′, and mixed probes with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxy-tetramethylrhodamine, TAMRA) and FAM-5′-TGACAGGGTTGATTCTCAGCCCTTCG-3′-TAMRA, FAM-5′-TGACCGGGTTGATTCTCAGCCCTTC-3′-TAMRA, and FAM-5′-TGACCGGGCTGATTCTCAGCCC TT-3′-TAMRA (Sigma-Aldrich Japan, Tokyo, Japan). Nucleic acid was extracted from 200 μL of serum and saliva and from 100 μL of 10 percent (wt/vol) fecal suspension in saline with kits (QIAamp MinElute virus spin kit, Qiagen K.K., Tokyo, Japan; and SMITEST R&D-EX, Medical & Biological Laboratories, Nagoya, Japan). Before extraction, the samples were centrifuged at 6000 × *g* at 4°C for 10 minutes; thereafter the clear supernatant was subjected to nucleic acid extraction. Before RT-PCR, RNA preparation of feces was diluted at 10 times with nuclease-free water to reduce the effect of inhibitors. Twenty microliters of nucleic acid sample was used for each reaction. Each 50 μL of reaction mixture contained 25 μL of 2× RT-PCR kit master mix (QuantiTect Probe RT-PCR kit, Qiagen), 0.5 μL of RT mix (QuantiTect Probe RT-PCR kit, Qiagen), 400 nmol per L each of forward and reverse primer, and 67 nmol per L each of three probes. RT-PCR mixture was incubated at 50°C for 30 minutes and at 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds, and 60°C for 1 minute utilizing a thermocycler (Applied Biosystems 7500, real time PCR system, Applied Biosystems, Tokyo, Japan). HEV nucleic acid testing (NAT) was performed individually. The analytical sensitivity of the HEV NAT was determined to be 25 (13-166) copies per mL (with 95% confidence interval) by logistic analysis. HEV viral load was determined from standard curves generated by using 10^1 to 10^7 copies of HEV RNA per reaction. The HEV quantitation standard was generated by transcribing HEV cDNA of HEV ORF2 region that was cloned into a plasmid (pCRII-TOPO, Invitrogen, Carlsbad, CA), using the in vitro transcription kit (MAXIscript T7 high-yield transcription kit, Ambion, Austin, TX). Purified plasmid DNA was linearized with *Hin*dIII restriction endonuclease and transcribed to yield 717-nucleotide-long RNA transcripts containing 75-nucleotide target sequence.

Phylogenetic analysis of HEV isolates

Entire or nearly entire sequences of HEV isolates were determined as previously described by Takahashi and coworkers.[4] The sequences were aligned together with reported HEV strains with a computer program (CLUSTAL W, Version 1.8).[17] A phylogenetic tree based on the nearly entire HEV RNA sequence was constructed by the neighbor-joining method,[18] and the final tree was obtained by a computer program (TreeView, Version 1.6.6).[19] Bootstrap values were determined by resampling 1000 times of the data sets. The nucleotide sequences isolates HRC-HE14C, JST-KitAsa04C, and JTC-Kit-FH04L reported in this study have been assigned DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB291965, AB291966, and AB291959, respectively.

Detection of serum anti-HEV

Samples were tested for immunoglobulin M (IgM)- and immunoglobulin G (IgG)-class antibodies against HEV using a commercial enzyme-linked immunosorbent assay kit (Viragent HEV-Ab, Cosmic Corp., Tokyo, Japan).[5,20]

Alanine aminotransferase testing

Alanine aminotransferase (ALT) testing was carried out using transaminase-HRII Nisseki/GPT (Wako Pure Chemical Industries Ltd, Osaka, Japan) on an automatic analyzer (ACA5400, Olympus Corp., Tokyo, Japan).

RESULTS

A lookback study of a causative blood donor

Blood from a 39-year-old Japanese male on September 20, 2004, was disqualified because of the elevated ALT level at 236 IU per L and tested for hepatitis viruses because of the abnormal ALT result. His blood sample turned out to be positive for the presence of HEV RNA at 4.8 log copies per mL as well as anti-HEV IgM and IgG and negative for the presence of any marker of hepatitis B virus (HBV) or hepatitis C virus (HCV). A lookback study revealed that his donated blood on September 6, 2004, 2 weeks before the last donation, was positive for the presence of HEV RNA at 3.1 log copies per mL and negative for the presence of IgM- or IgG-class anti-HEV. The HEV isolate, HRC-HE14C, was classified as genotype 4 of a Japan-indigenous strain (Fig. 1). The blood (platelet [PLT] concentrate) donated on

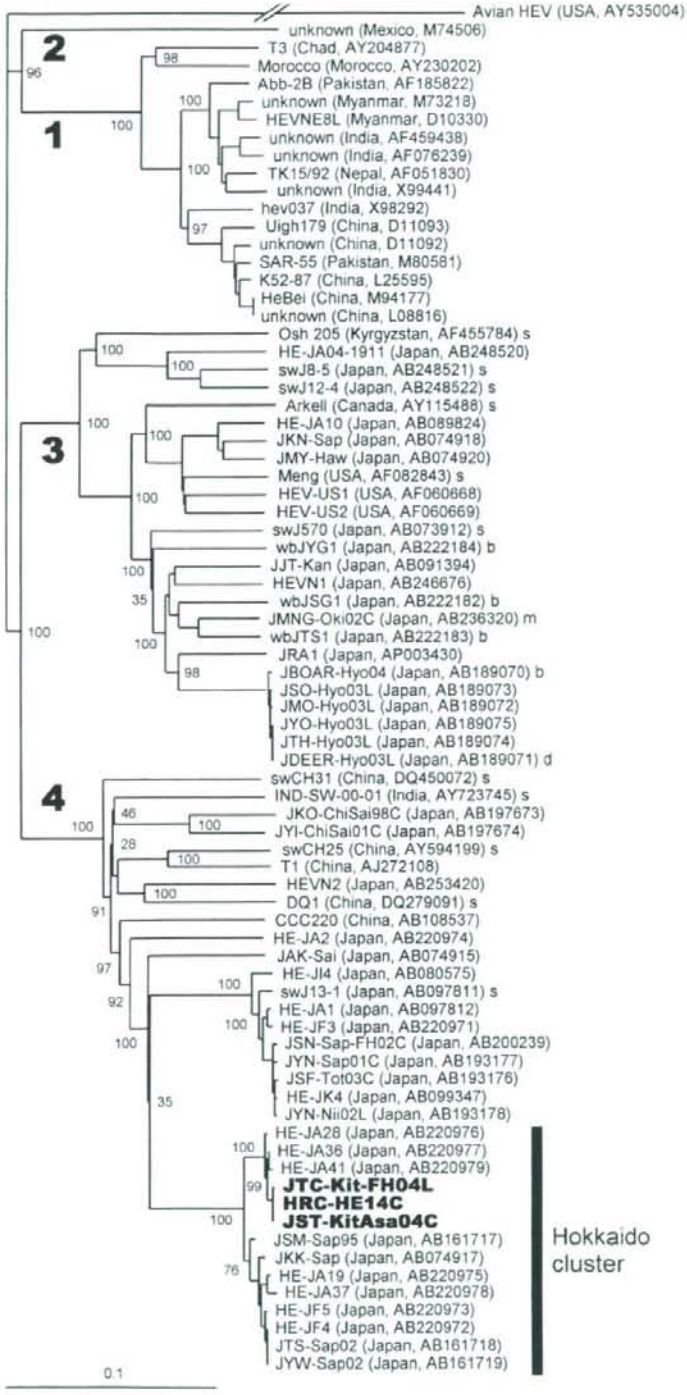

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the entire or nearly entire sequences of HEV genome of 77 isolates using an avian HEV (AY535004) as an outgroup. After the isolate name, the name of the country where the strain was isolated and accession numbers are shown in parentheses. The numbers 1, 2, 3, and 4 in bold indicated HEV genotypes. The 3 isolates HRC-HE14C from the causative donor, JST-KitAsa04C from the patient, and JTC-Kit-FH04L from the donor's father are indicated in bold. The letters "s," "b," "d," and "m" after parentheses denote HEV isolates from farmed pig, wild bore, wild deer, and mongoose, respectively. A vertical bar represents a cluster consisting of strains indigenous to Hokkaido, Japan. Bootstrap values are indicated for the major nodes as a percentage of the data set obtained from 1000 resamplings.

September 6 was released because it showed normal ALT and passed all the current blood screening tests. Transfusion was carried out 3 days after the blood donation, and the total amount of HEV in the PLT concentrate was estimated to be approximately 5.4 log copies. He was asymptomatic and did not feel tired or febrile in the periods near the two occasions of blood donation.

A minioutbreak of HEV infection in family members of the causative donor

Besides the causative donor, HEV RNA was detected in the blood of his 69-year-old father, who developed acute hepatitis on September 14, 2004, and finally died of fulminant hepatitis on October 14. Retrospective analysis of the father's blood sample taken on September 24, 41 days after the dining, revealed that the HEV strain, JTC-Kit-FH04L, was genotype 4. HEV RNA sequence analysis of the HEV isolates from the causative donor and his father showed only 9-nucleotide differences of 6588 nucleotides, suggesting that the two strains were extremely close but not identical (Fig. 1).

By retroactive interviewing, it was revealed that the causative donor and his 12 relatives gathered to enjoy grilled meats

including pig liver and intestines at a barbecue restaurant on August 14, 2004.[21] Blood samples from the relatives were tested for HEV markers with informed consent. Seven of the family members who ate grilled pig liver and/or intestines had IgM- and/or IgG-class anti-HEV in the blood samples taken 37 to 92 days after the barbecue party. Retrospectively, in the previous 6 months or more, dining out at that restaurant was the only occasion all the 13 relatives had eaten together.

Clinical course of the patient

It was confirmed that the PLT concentrate (approx. 200 mL) contaminated with HEV was transfused to a 64-year-old Japanese male patient with non-Hodgkin's lymphoma on September 9, 2004, as shown Day 0 in Fig. 2. The patient had been treated with autologous peripheral blood stem cell transplantation accompanied with heavy chemotherapy since July 30, 2004. In the first 3 weeks after the transfusion, liver function tests sustained to be normal. On Day 22, the ALT level increased transiently at 67 IU per L, and HEV was detected in serum. While the ALT level returned to normal, the viral load in serum showed an exponential increase. Levels of aspartase aminotransferase (AST) and ALT took an upward turn on Day 41. There was no evidence for acute infection of hepatitis A virus, HBV, HCV, cytomegalovirus, or Epstein-Barr virus. He was diagnosed as acute hepatitis E. On Day 45, he was referred to the liver unit of Teine Keijinkai Hospital to treat presumed developing acute hepatitis E. Despite antiviral therapy with interferon (IFN) from Day 45, 2′,5′-oligoisoadenylate synthetase in serum never showed apparent increase and no obvious decrement of viral load had obtained (Fig. 2A). Levels of AST and ALT indicated creeping increase to reach highest levels of 903 and 673 IU per L on Day 59, respectively (Fig. 2C). The treatment was switched from IFN to predonisolone (PSL) in expectation of its anti-inflammatory effect. From Day 59 after induction of PSL treatment, AST and ALT showed rapid decrease and improvement of prothrombin time was observed (data not shown). Dosage of PSL was tapered gradually and discontinued on Day 113. Soon after anti-HEV IgG emerged on Day 67, HEV load in the serum sample had declined rapidly, although anti-HEV IgM in the serum sample remained negative (Figs. 2A and 2B). The levels in aminotransferases were normalized after Day 95 (Fig. 2C). The HEV strain JST-KitAsa04C detected in the patient was genotype 4 and its entire sequence analysis showed only a 1-nucleotide difference of 7255 nucleotides, suggesting the two isolates were identical (Fig. 1).

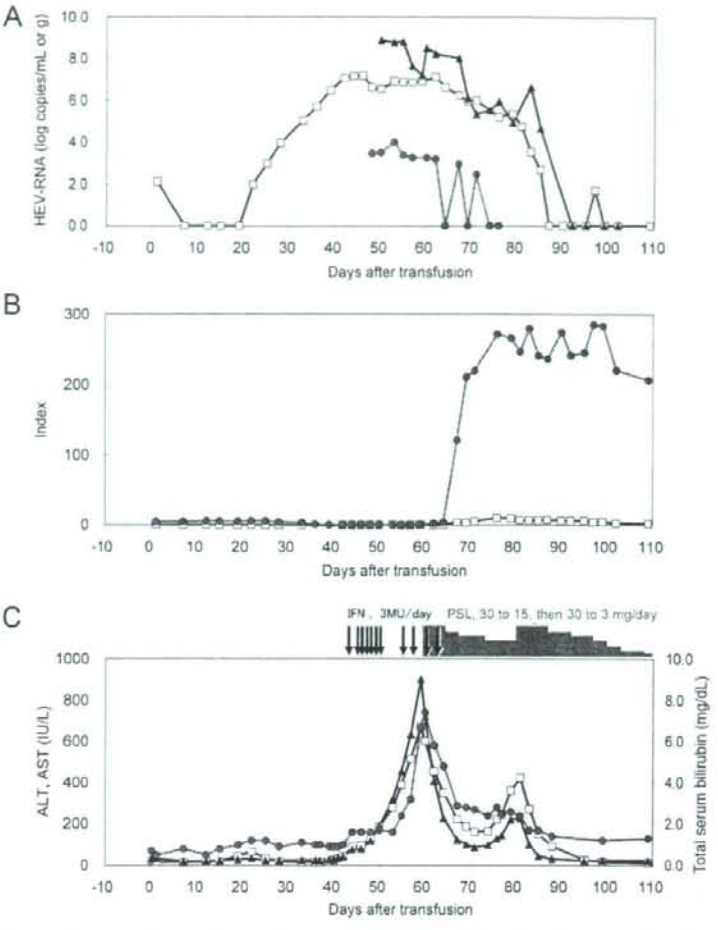

Fig. 2. Clinical course of transfusion-transmitted hepatitis E with kinetics of (A) HEV RNA, (B) serologic, and (C) biochemical markers after transfusion. The patient had transfusion of PLT concentrates contaminated with HEV on Day 0. (A) HEV RNA load was represented as log copies per mL of serum (□) or saliva (●) or per g of feces (▲). There were no data between Day 0 and Day 44 in feces and saliva. (B) Cutoff values of anti-HEV IgM (□) and IgG (●) antibodies are 30 and 13, respectively. (C) Medications were administered with IFN-α from Day 43 through Day 62 and with PSL from Day 59 through Day 112. (□) ALT; (▲) AST; (●) total serum bilirubin.

Serial quantitative changes of HEV load in serum, saliva, and feces of the patient

HEV RNA and anti-HEV were measured for every serum sample before and after the transfusion. In addition, HEV loads were also assessed prospectively for feces and saliva after his transference to the liver unit on Day 45. Any marker for HEV was not detected in serum sampled 37 days before the transfusion. A small amount of HEV RNA was transiently detected in his serum on Day 1, the next day of the transfusion. After the reappearance on Day 22, HEV RNA showed exponential increment with doubling every 29 hours and reached the peak level of 7.2 log copies per mL on Day 44. Beyond its plateau phase lasting 3 weeks, viral load revealed gradual decline over 2 weeks and thereafter decreased promptly. HEV viremia had been finally sustained for 63 days. HEV RNA remained detectable up to Day 97 in serum, Day 71 in saliva, and Day 85 in feces. Peak levels of HEV RNA were found on Day 53 in saliva at 4.0 log copies per mL and on Day 50 in feces at 8.9 log copies per g, respectively. HEV RNA was no longer detectable after Day 99 (Fig. 2A).

DISCUSSION

In Japan, a nonendemic country for hepatitis E, HEV infection is occurring more frequently than previously recognized. The prevalence of anti-HEV IgG in healthy Japanese persons ranged from 1.9 to 14.1 percent, depending on the geographic area,[20] and the prevalence of HEV RNA among Japanese blood donors with ALT level of at least 201 IU per L was 2.8 percent.[21] The risks of transfusion transmission of HEV might be low; however, five molecularly confirmed cases of transfusion-transmitted HEV infection have been reported in nonendemic countries so far.[12-16] In none of them, HEV infection routes of the causative donors are known. In this report, we have described the first case that the infection route of donor is clarified as zoonotic food-borne. The conclusion is based mainly on two observations.

First, by the epidemiologic study, the donor was determined to be infected in a minioutbreak of HEV infection in the context of food-borne transmission. Six of the 13 relatives who dined out together were positive for the presence of HEV RNA and/or IgM anti-HEV in their serum samples obtained 37 to 92 days after dining at the restaurant (Appendix 1). As for 4 relatives who were positive for the presence of IgM anti-HEV, HEV viremia might have transiently occurred without any symptom and had subsided by the time when blood samples were taken. Since IgM anti-HEV are regarded as the markers of acute HEV infection besides HEV RNA,[10] these facts strongly suggest that family members had recently become infected with HEV probably at the same time and remained asymptomatic. The party at the barbecue restaurant was the only opportunity all the 13 members had eaten together in the estimated period of HEV infection, 2 to 10 weeks.[22,23] Although it was difficult to identify the source of infection because no meat was left, they ingested various kinds of pig meats including liver and intestines, according to the replies to the questionnaire from the family members.[24] From this retrospective research, it is strongly suspected that the family members shared the motive of infection with HEV by ingestion of pig liver and intestines. In Japan, HEV has been isolated from farmed pigs,[9,25] wild deer,[8,26,27] and wild boar[10,11,26,27] as well as humans and recent studies also indicated that HEV is moderately resistant to heat inactivation.[28,29] Some reports suggest that a number of hepatitis E cases in Japan may be via a zoonotic food-borne route.[8-11,25-27,30]

Second, a single transmission route of HEV in this minioutbreak is corroborated by molecularly confirmed facts. From full-length sequence analysis, HEV RNAs detected in the donor and recipient were identical and closely related to that in his father. Among the strains of genotype 4 indigenous to Hokkaido, Japan, these three strains were segregated into a distinct cluster with a bootstrap value of 99 percent in a phylogenetic tree based on the entire or nearly entire sequences of HEV genome. Moreover, when comparing 412-nucleotide sequences (nucleotides 5985-6396 of HRC-HE14C) of ORF2 region, where many sequences of Japanese swine HEV are retrievable in DDBJ/EMBL/GenBank nucleotide sequence databases, high similarity (409/412 nucleotides, 99.3%) was observed between the HEV sequences derived from the causative donor and his father and strain swJL145 (AB105902),[9] which was detected in pig liver sold at a drug store in Hokkaido, Japan.

To date, in acute hepatitis E including transfusion transmission cases, dynamic relationships between infection markers for HEV and disease progression throughout the course from HEV transmission to convalescence of disease have not been demonstrated. This is the first case of acute hepatitis E, in which HEV kinetics in serum as well as in feces and saliva were described by using quantitative RT-PCR for HEV RNA from transfusion up to the end of viremia accompanied by disease progression, and the emergence and increase of anti-HEVs. In the current case, HEV viremia had lasted for 9 weeks or more and viral load reached its peak 15 days before the peak of aminotransferase level and died out promptly right after the appearance of anti-HEV IgG on Day 67. The results led us to understand the chronologic relationship between preceding viremia and after emergence and increase of anti-HEV.

Besides serum, the kinetics of HEV load in feces and saliva were concomitantly observed for the first time in hepatitis E in humans. After the transmission, HEV RNA remained detectable until Day 71 in saliva and Day 85 in feces. Among sera, saliva, and feces, every time point at peak viral loads resembled each other, 50 to 60 days after transmission. These facts may indicate that viral loads in

saliva and feces would also reflect viremia state. In addition, the results for saliva suggest that besides fecal-oral route, oral-oral transmission manner can be another route of human-to-human infection of HEV.

Soon after the transference to liver unit in the hospital, IFN-α therapy was started against HEV infection, indicating the exponential increase of viral load in sera. The levels in 2',5'-oligoadenylate synthetase, however, induced by IFN and regarded as a predictive marker for favorable IFN efficacy,[31] did not show sufficient increase in serum (data not shown), and HEV load monitored concomitantly indicated no actual decrement during treatment. Thereafter, single-nucleotide polymorphisms in markers predicting the therapeutic efficacy of IFN, such as mannose-binding lectin,[32] MxA,[33] LMP7,[34] and osteopontin,[35] were examined, and all of them did not show the phenotype associated with favorable efficacy of IFN (data not shown).

Throughout his clinical course, no distinct positive result for IgM anti-HEV was observed. It is possible that the concentration of IgM anti-HEV was too low to be detected by the method we used. In fact, some of his samples showed equivocal reaction. Furthermore, underlying disease and the preceding treatment including autologous peripheral blood stem cell transplantation and large dosage chemotherapy might have led the patient to an immunocompromised state that responds inadequately for HEV infection. In fact, both serum levels in IgG and IgM had been indicated consistently less than lower limitation of normal ranges in the entire course (data not shown).

We should note that the present case was not revealed if the two practices had not been introduced, which are not widespread outside Japan. They are ALT screening and donor blood sample repository system. As a safety measure, the Japanese Red Cross Blood Center introduced ALT testing for a surrogate marker for non-A, non-B hepatitis virus infection. Because ALT testing contributes little for HCV infection after HCV antibody testing started, ALT screening has been discontinued in the United States and some other countries. Although the cutoff value may need to be reevaluated, the current case suggests that ALT testing may contribute to excluding blood with the presence of HEV. On the other hand, the Japanese Red Cross has established storing repository samples of all donations since 1996. Blood samples are collected from each donation and stored for 10 years at –30°C to investigate for lookback study such as the suspected cases of transfusion-transmitted infection and alloantibodies for TRALI. This system plays a very important role in the hemovigilance system in Japan.[36,37]

In the present case of transfusion-transmitted acute hepatitis E, the infection route in the blood donor was, for the first time, clarified to be zoonotic food-borne manner. In addition, the entire course including incubation period and disease progression in acute HEV infection was followed by serologic and virologic markers, and the patient was treated by monitoring them. To our knowledge, this is the first report for acute HEV infection in humans, in which various infection markers were prospectively monitored simultaneously with disease progression, excepting experimental hepatitis E in a volunteer.[38]

Our data suggest that hepatitis E is likely caused by consumption of contaminated pig meat, and there is a risk of transfusion transmission of HEV in Japan. The most effective preventive measure to reduce the risk of blood-borne transmission is to screen the blood supply for HEV or to implement pathogen inactivation. The epidemiology and the transfusion-related risks for HEV infection have not been fully understood in industrialized countries including Japan. We are undertaking epidemiologic studies of HEV infection in Japanese blood donors and a feasibility study of NAT screening for HEV in Hokkaido, Japan.

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APPENDIX 1

HEV infection markers in the 13 family members who participated in the dinner on August 14, 2004

Number*	Age (years)	Sex	Days after Aug 14, 2004	ALT (IU/L)	HEV markers		
					RNA (10^6/mL)	IgM† (index)	IgG‡ (index)
1	39	Male	23	27	+(3.1)	−(3.4)	−(2.0)
			37	236	+(4.8)	+(60.4)	+(14.2)
			49	70	+(2.1)	+(269.5)	+(154.7)
			53	44	−	+(257.8)	+(150.5)
			77	20	−	+(174.6)	+(163.0)
2	69	Male	41	1511	+(2.6)	+(187.2)	+(271.4)
3	43	Male	92	34	−	+(174.7)	+(297.7)
4	68	Male	79	15	−	+(51.7)	+(283.3)
5	37	Female	79	13	−	+(110.9)	+(90.3)
6	15	Male	90	17	−	+(63.3)	+(250.6)
7	58	Female	79	25	−	−(4.0)	+(25.9)
8	67	Female	79	15	−	−(1.4)	−(12.9)
9	38	Female	89	12	−	−(6.1)	−(1.1)
10	15	Male	77	19	−	−(0.3)	−(0.5)
11	14	Male	77	19	−	−(7.5)	−(0.3)
12	46	Male	90	15	−	−(2.2)	−(0.4)
13	6	Female	90	15	−	−(26.6)	−(1.1)

Data shown were originally reported by Kato et al.[24] without describing quantitative test results of antibodies and viral RNA and follow-up data of the causative donor.
* Number 1 is the causative donor; Number 2 is the donor's father and died of hepatitis E; others are their relatives.
† Positive ≥30 index.
‡ Positive ≥13 index.

Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

Keisuke Kohga,[1,5] Tetsuo Takehara,[1,5] Tomohide Tatsumi,[1] Kazuyoshi Ohkawa,[1] Takuya Miyagi,[1] Naoki Hiramatsu,[1] Tatsuya Kanto,[1] Tsutomu Kasugai,[2] Kazuhiro Katayama,[2] Michio Kato[3] and Norio Hayashi[1,4]

[1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; [2]Department of Gastroenterology and Hepatology, Osaka Koseinenkin Hospital, 4-2-27 Fukushima, Fukushimaku, Osaka, Osaka 553-0003; [3]Department of Gastroenterology and Hepatology, National Hospital Organization, Osaka National Hospital, 2-1-14 Hoenzaka, Chuoku, Osaka, Osaka 540-0006, Japan

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Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial emboli-zation therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

MHC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.[1] In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.[2–5] The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.[6] Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.[7] In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.[8–10] The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.[4] In addition, sera from those patients can downregulate NKG2D expression *in vitro*.[5,11] These data suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.[12,13]

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.[14,15] With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.[16] We reported previously that soluble MICA could be detected in sera of HCC patients.[17] However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

*To whom correspondence should be addressed.
E-mail: hayashin@gh.med.osaka-u.ac.jp
[5]Keisuke Kohga and Tetsuo Takehara contributed equally to this work.
Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

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Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH		0/5/	2/1/	4/0/
AIH/PBC/others		2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.
*$P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2-test; **$P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; ***$P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; ****$P < 0.05$ vs cirrhosis by χ^2-test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.[18] Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.[16]

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparafinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the $\alpha 1$ and $\alpha 2$ domains of MIC molecules shared by both MICA and MICB.[2] To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t-test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

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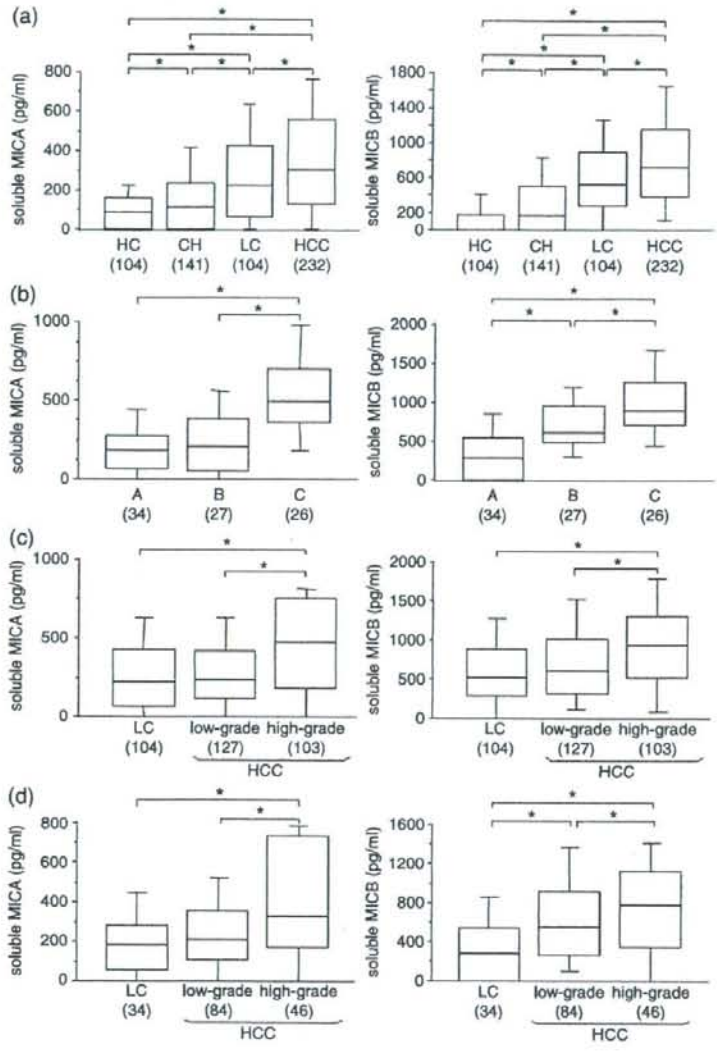

Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child–Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child–Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. $*P < 0.05$ by Kruskal–Wallis test and post hoc Steel–Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhosis patients were stratified based on Child–Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child–Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

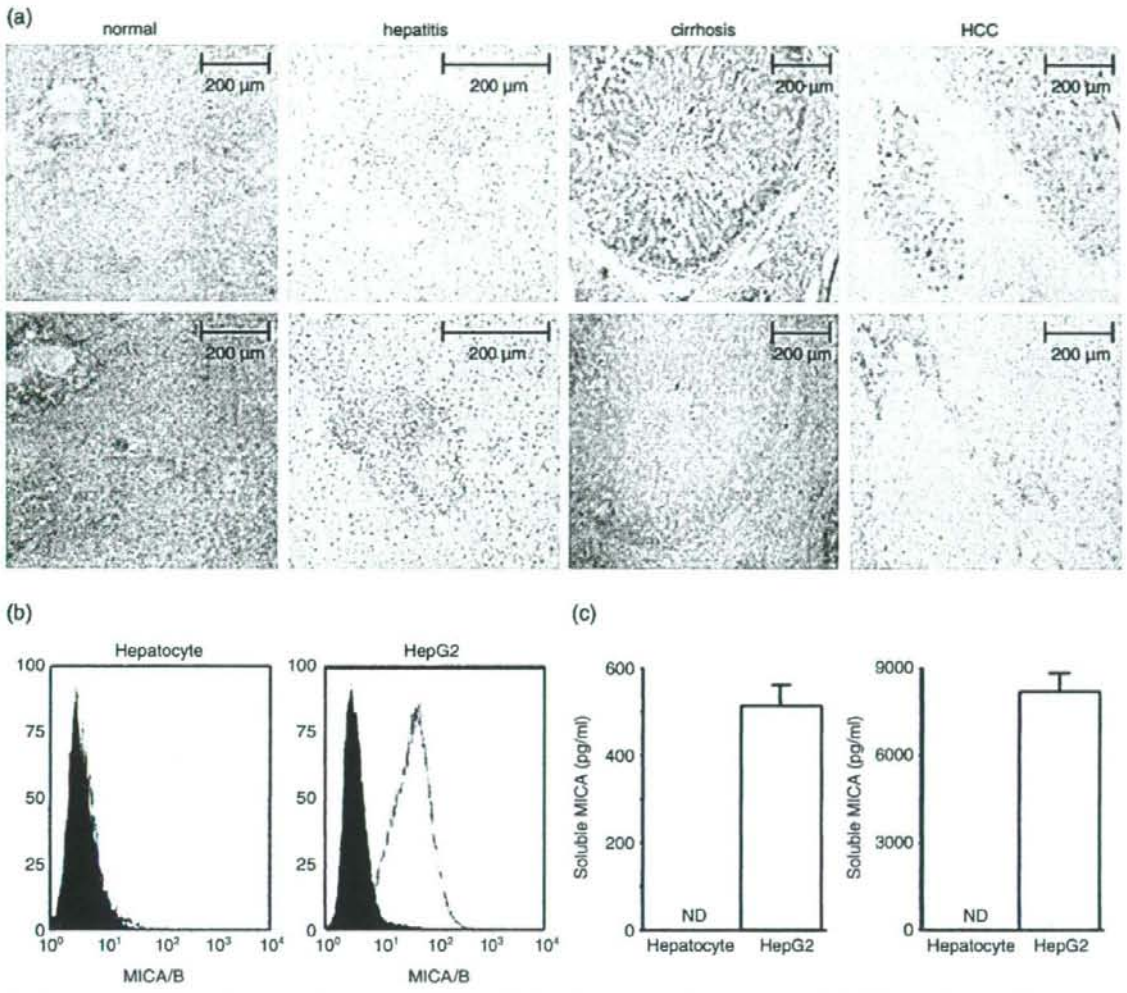

Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MICB released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.[3] Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

Downregulation of soluble MICA levels by TAE. The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

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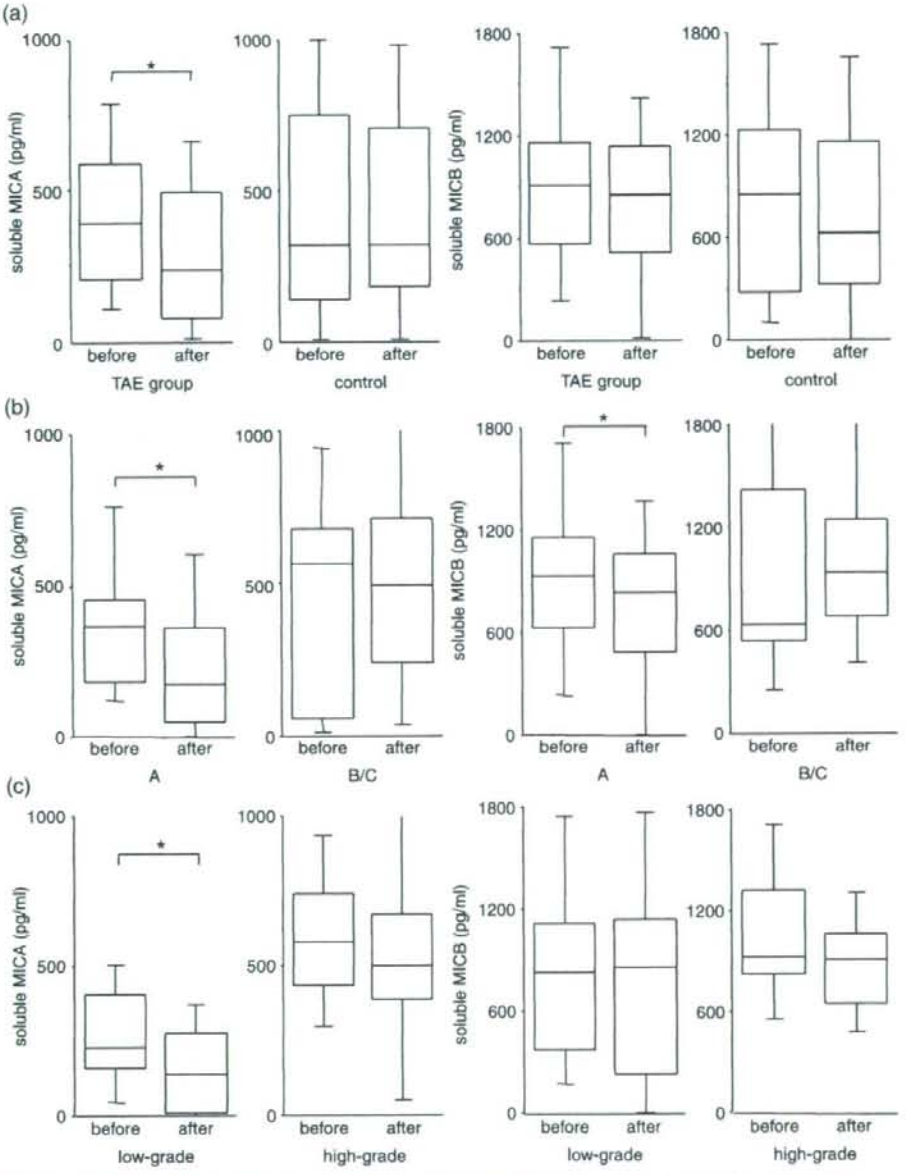

Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divide into two groups: Child–Pugh A ($n = 29$) and Child–Pugh B and C ($n = 9$). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ($n = 24$) and high-grade HCC ($n = 14$). *$P < 0.05$ by paired t-test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble MICA/B, TAE-treated patients were divided according to their Child–Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child–Pugh A patients but not in Child–Pugh B and C patients (Fig. 3b). Interestingly, Child–Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child–Pugh B and C patients did not. As for tumor stage, a significant decrease in

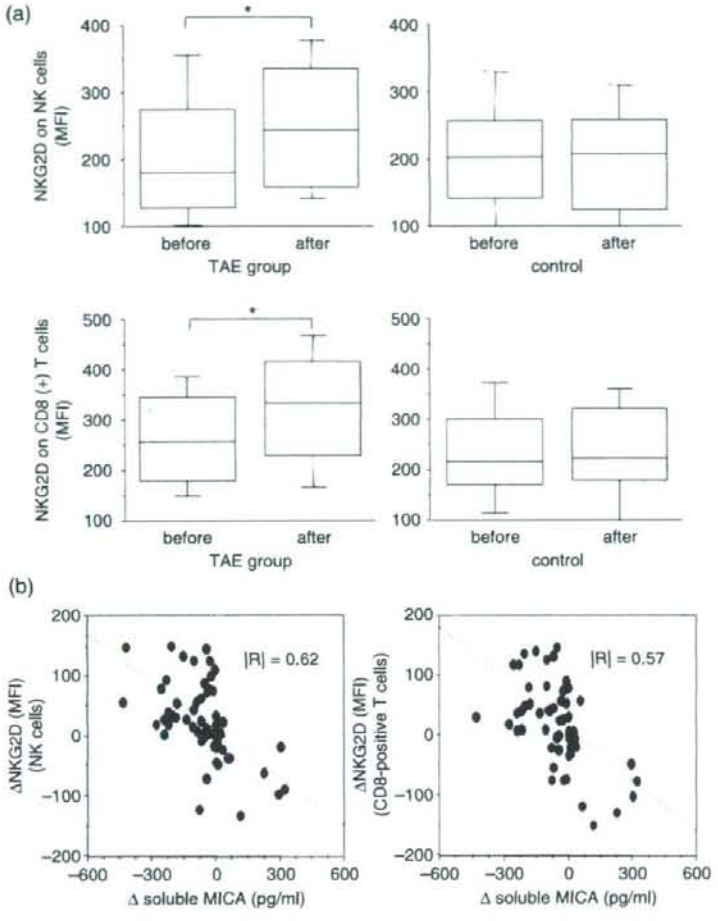

Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). $*P < 0.05$ by paired *t*-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

Upregulation of NKG2D expression by TAE. The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.[12] However, little is known about soluble MICA/B in the premalignant condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.[12,13] They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.[2] In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed

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hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.[19] It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.[20] It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.[16] To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child–Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child–Pugh A patients than Child–Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child–Pugh A patients in our cohort showed a larger decease in α-fetoprotein levels after TAE therapy than Child–Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

It is generally speculated that soluble MICA/B produced from tumors may deactivate NKG2D-mediated immune responses.[8,9] *In vitro* experiment indicates that soluble MICA could down-regulate NKG2D expression and effector cell function. However, the regulation by soluble forms of NKG2D ligands would be more complicated *in vivo*. First, soluble forms of NKG2D ligands could be produced not only from malignant tissues but also from non-malignant tissues, as shown in the present study. Second, MHC-encoded MICA/B may not be the sole family of proteins serving as NKG2D ligands. Non-MHC-encoded UL16-binding proteins also act as NKG2D ligands and were very recently found to be cleaved proteolytically from tumor cells.[21] The present study provides evidence that soluble MICA is derived, at least in part, from HCC and regulates NKG2D expression on NK and CD8-positive T cells. Although several species of soluble NKG2D ligands may exist in the circulation, the present study suggests that soluble MICA regulates NKG2D expression directly in cancer patients.

In conclusion, soluble MICA and MICB are significantly increased in the sera of patients not only with HCC but also with chronic liver disease. Soluble MICA/B increases together with the progression of liver disease as well as the tumor. Therapeutic intervention for HCC leads to reduction of soluble MICA levels in association with upregulation of NKG2D on immune cells, offering *in vivo* evidence of soluble MICA regulating NKG2D expression. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated immune response even if some of the soluble NKG2D ligands are produced from non-cancerous premalignant tissues.

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METABOLISM, CANCER AND GENETICS

Molecular basis for the synergy between alcohol and hepatitis C virus in hepatocarcinogenesis

Kazuhiko Koike, Takeya Tsutsumi, Hideyuki Miyoshi, Seiko Shinzawa, Yoshizumi Shintani, Hajime Fujie, Hiroshi Yotsuyanagi and Kyoji Moriya

Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Kazuhiko Koike

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Correspondence
Professor Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-tky@umin.ac.jp

Abstract

Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). In addition, heavy alcohol use has been linked with earlier progression to HCC in chronic hepatitis C patients. However, in the pathogenesis of HCV-associated HCC, it still remains controversial as to whether the virus plays a direct or an indirect role, and as to how alcohol operates in the acceleration of HCC development. Several studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, although other factors such as continuous inflammation or environmental factors seem also to play a role. The downstream events of the HCV core protein expression in the transgenic mouse HCC model are segregated into two pathways. One is the augmented production of oxidative stress in the absence of inflammation along with the attenuation of some scavenging systems in the putative preneoplastic stage with steatosis in the liver. The other pathway is the alteration in cellular gene expression and intracellular signaling, including the mitogen-activated protein kinase cascade. The combination of these pathways would explain the unusually high incidence and multicentric nature of HCC development in HCV infection. In addition, alcohol feeding in this animal model further activated the two pathways synergistically with HCV, leading to an earlier development of HCC. Such a synergy would reveal the molecular basis for the acceleration of HCC development by alcohol in HCV infection.

Introduction

Hepatitis C virus (HCV) infects approximately 170 million people persistently worldwide, and induces a spectrum of chronic liver diseases, from chronic hepatitis, cirrhosis to hepatocellular carcinoma (HCC).[1] HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. It impacts the medical, sociological and economic domains of society. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%,[2] resulting in the development of HCC in nearly 90% of HCV-associated cirrhosis patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection (i.e. development of HCC in a multicentric fashion and a very high incidence), are not common in other malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection is therefore imminently required for the prevention of HCC.

However, alcohol has been known as an accelerating factor in the development of HCC in persistent HCV infection.[3–5] The pattern of the risk for HCC due to alcohol intake shows a continuous dose-effect curve without a definite threshold, although most studies have found that HCC risk increased only for alcohol consumption above 40–60 g of ethanol per day. Some evidence supports a positive interaction of alcohol intake, probably with HCV infection and possibly with HBV infection.[3] Synergistic interactions on the additive model were observed between heavy alcohol consumption and chronic hepatitis virus infection and diabetes mellitus.[4] However, it is unclear how alcohol causes the acceleration of HCC development in HCV infection.

How does HCV contribute to hepatocarcinogenesis?

How HCV is involved in hepatocarcinogenesis is not yet clear, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.[1,6,7] HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence force us to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence or is there a multicentric nature in HCV infection?

The other role of HCV would be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. This background and reasoning led to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing HCV genes into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. In fact, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, *in vivo*, by transgenic mouse technology.

Transgenic mouse studies revealed an *in vivo* oncogenic activity of HCV core protein

Transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes from cDNA of the HCV genome of genotype 1b.[8,9] Three different transgenic mouse lines were established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages.[9] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[10,11] The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, and the intrahepatic level of the core protein is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[12] Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[13–15] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

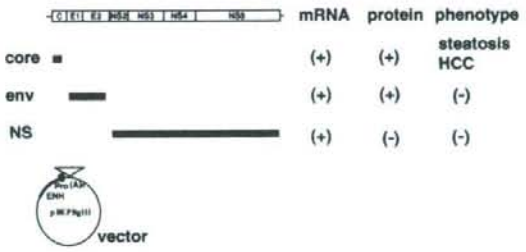

Figure 1 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different types of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene developed hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes did not develop HCC. env, envelope genes; NS, non-structural genes.

Oxidative stress overproduction and MAPK activation as consequences to the core protein expression in the liver

It is difficult to determine the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature in the localization of the core protein in hepatocytes: while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[9,16] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[17,18] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. In addition, analysis of the anti-oxidant system revealed that some anti-oxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice; hemeoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione *S*-transferase levels are increased and enhanced by iron overloading (S Shinzawa *et al.*, unpubl. data, 2007). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the anti-oxidant system, which may explain the mechanism underly-

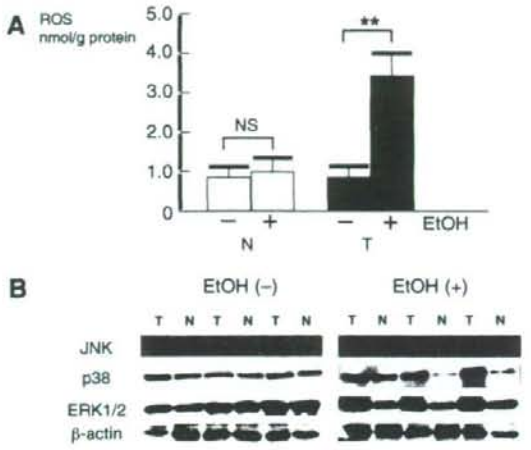

Figure 2 Alcohol administration enhances oxidative stress production and mitogen-activated protein kinase (MAPK) pathway activation in a synergistic fashion with hepatitis C virus (HCV) core protein. Administration of 5% alcohol for 3 weeks provoked an induction of reactive oxygen species (ROS) in HCV core gene transgenic mice, whereas it induced only a marginal increase in control mice, showing a synergy between the HCV core protein and ethanol in inducing ROS. Only the c-Jun N-terminal kinase (JNK) pathway is activated in the core gene transgenic mice before hepatocellular carcinoma (HCC) development, but feeding 5% alcohol for 3 weeks activated the other two pathways, p38 and ERK1/2, which was not observed in control mice. Thus, combining the effect of ethanol to that of the core protein resulted in the activation of all the MAPK pathways, among which only JNK was activated by the action of HCV core protein only in the absence of ethanol. ERK, extracellular signal-regulated kinase; EtOH, ethanol; N, non-transgenic control mouse; NS, statistically not significant; T, transgenic mouse. $^{**}P < 0.01$.

ing the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged any longer by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic difference from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.[9,17] The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[19] Hepatic steatosis in hepatitis C, which is also attributed to the action of the core protein,[8] may work as fuel for oxidative stress overproduction.[18,20,21]

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated.[22] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that play pivotal roles in cell proliferation and lipid metabolism.[23] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.[22,24] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and confers an advantage for cell proliferation to hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28γ (PA28γ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.[25,26]

Such an effect of the core protein on the MAPK pathway, in combination with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C.

Molecular basis for the synergy between alcohol and HCV infection in hepatocarcinogenesis

As described above, the production of oxidative stress is increased in the liver of aged HCV core gene transgenic mice in the absence of inflammation. In young mice, the increase in oxidative stress is apparently marginal. However, feeding 5% ethanol to mice for 3 weeks induced ROS in the liver of core gene transgenic mice, whereas it induced only a minimal increase in control mice, demonstrating a synergy between the core protein and ethanol in inducing ROS (Fig. 2a).[17] In contrast, only the JNK pathway is activated in the core gene transgenic mice before HCC development, but feeding 5% ethanol for 3 weeks activated the other two MAPK pathways, p38 and ERK1/2 in the core gene transgenic mice, the activation of which is not present in control mice (Fig. 2b). Thus, combining the effect of ethanol to that of the core protein provoked the activation of all the MAPK pathways, affording advantage to cell proliferation.[24]

In a long-term observation experiment, feeding 2% ethanol to the core gene transgenic mice for 9 months resulted in the acceleration of HCC development (Moriya K *et al.*, unpubl. data, 2007). Screening by the high-throughput immunoblot analysis revealed differential expression of proteins in the liver with or without ethanol feeding; some proteins, the levels of which were either increased or decreased by the effect of the core protein, such as Rho GTPase activating protein (GAP) or caspase-8, are down- or upregulated by the effect of ethanol feeding.

In summary, we postulate that the induction of oxidative stress, together with the activation of MAPK cascade, followed by AP-1 activation and cyclin D1 overexpression, plays a pivotal role in the development of HCC (Fig. 3). Alterations in cellular gene expressions, such as TNF-α or suppressor of cytokine signaling-1, and the

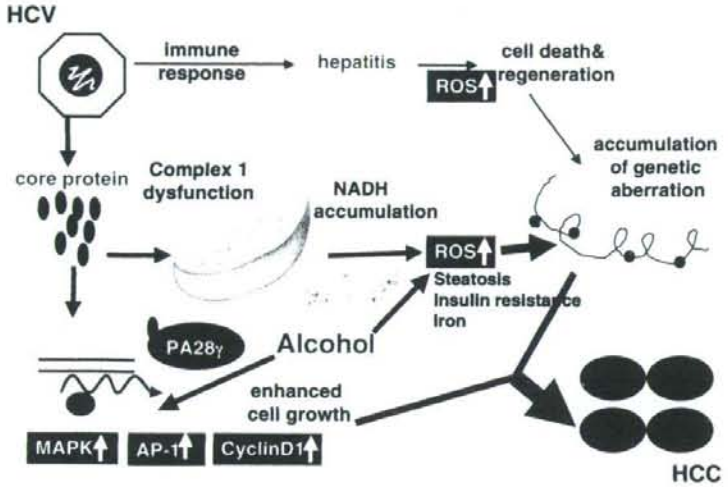

Figure 3 Molecular pathogenesis of hepatocellular carcinoma (HCC) development in hepatitis C virus (HCV) infection in association with alcohol. We postulate that induction of oxidative stress through the dysfunction in the mitochondrial electron transfer system, together with alterations in cellular gene expressions and the intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade, play a pivotal role in the development of HCC. Alcohol activates both of these pathways and augments the development of HCC in HCV infection. AP-1, activating protein-1; NADH, nicotinamide adenine dinucleotide; PA28γ, proteasome activator 28γ; ROS, reactive oxygen species; SOCS-1, suppressor of cytokine signaling-1; TNF-α, tumor necrosis factor-α.

presence of steatosis and insulin resistance are co-accelerators to hepatocarcinogenesis in HCV infection. Finally, alcohol augments both of these pathways that are activated by the core protein, and further enhance the development of HCC in HCV infection (Fig. 3).

Conflict of interest

No conflict of interest has been declared by the authors.

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