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Potent and selective inhibition of hepatitis C virus replication by novel phenanthridinone derivatives

Mohammed T.A. Salim [a], Hiroshi Aoyama [b,1], Kazuyuki Sugita [b], Kouichi Watashi [c], Takaji Wakita [c], Takayuki Hamasaki [a], Mika Okamoto [a], Yasuo Urata [d], Yuichi Hashimoto [b], Masanori Baba [a,*]

[a] Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan
[b] Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan
[c] Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[d] Oncolys BioPharma Inc., Tokyo 105-0001, Japan

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ABSTRACT

A number of novel phenanthridinone derivatives were examined for their inhibitory effect on hepatitis C virus (HCV) replication in Huh-7 cells harboring self-replicating subgenomic viral RNA replicons with a luciferase reporter (LucNeo#2). The activity of compounds was further confirmed by inhibition of viral RNA copy number in different subgenomic and full-genomic replicon cells using real-time reverse transcription polymerase chain reaction. Among the compounds, 4-butyl-11-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-7-methoxy-[1,3]dioxolo[4,5-*c*]phenanthridin-5(4*H*)-one (HA-719) was found to be the most active with a 50% effective concentration of 0.063 ± 0.010 μM in LucNeo#2 cells. The compound did not show apparent cytotoxicity to the host cells at concentrations up to 40 μM. Western blot analysis demonstrated that HA-719 reduced the levels of NS3 and NS5A proteins in a dose-dependent fashion in the replicon cells. Interestingly, the phenanthridinone derivatives including HA-719 were less potent inhibitors of JFH1 strain (genobtype 2a HCV) in cell-free virus infection assay. Although biochemical assays revealed that HA-719 proved not to inhibit NS3 protease or NS5B RNA polymerase activity at the concentrations capable of inhibiting viral replication, their molecular target (mechanism of inhibition) remains unknown. Considering the fact that most of the anti-HCV agents currently approved or under clinical trials are protease and polymerase inhibitors, the phenanthridinone derivatives are worth pursuing for their mechanism of action and potential as novel anti-HCV agents.

1. Introduction

Hepatitic C virus (HCV) infection is a worldwide problem. More than 130 million individuals are infected with this virus, and 3–4 million are newly infected every year. In general, HCV infection proceeds to chronic infection [1], which often induces liver cirrhosis and hepatocellular carcinoma [2] liver transplantation is the only way to rescue patients with the end-stage liver disorders caused by HCV infection [3]. Protective vaccines are not available so far, and pegylated interferon (PEG-IFN) and the nucleoside analog ribavirin are the standard treatment for HCV infection [4–6]. However, many patients cannot tolerate the serious side effects of PEG-IFN and ribavirin. Therefore, the development of novel agents with better efficacy and tolerability is still mandatory.

HCV is an enveloped virus belonging to the hepacivirus genus of the family *Flaviviridae* [7,8]. The viral genome consists of positive sense single RNA coding a polyprotein cleaved by viral and host proteases into four structural and six non-structural proteins. Non-structural proteins are involved in the replication of HCV genome [9]. The discovery of effective anti-HCV agents was greatly hampered by the lack of cell culture systems that allowed robust propagation of HCV in laboratories. However, the development of HCV RNA replicon systems [10] and recent success in propagating infectious virus particles in vitro have provided efficient tools for screening new antiviral agents against HCV replication [11,12]. Furthermore, replicons containing a reporter gene, such as luciferase and green fluorescence protein, have provided fast and reproducible screening of a large number of compounds for their antiviral activity [13–15].

Currently, two NS3 protease inhibitors, teraprevir and boceprevir, have been licensed and a considerable number of novel anti-HCV agents are under clinical trials [16,17]. Most of them are directly acting inhibitors of NS3 protease or NS5B polymerase. However, the

* Corresponding author. Fax: +81 99 275 5932.
E-mail address: m-baba@m2.kufm.kagoshima-u.ac.jp (M. Baba).
[1] Present address: School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan.

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emergence of HCV mutants resistant to most of these agents has also been reported [18]. To circumvent the drug-resistance, it seems necessary to use more than two directly acting drugs targeting different molecules for inhibition of viral replication [19]. Thus, in addition to the protease and polymerase inhibitors, novel compounds with a unique mechanism of action are highly desired.

We have recently identified some compounds with a novel phenanthridinone structure as moderate inhibitors of HCV replication [20]. This prompted us to synthesize a number of phenanthridinone derivatives and investigate their anti-HCV activity. After optimization of chemical structures, we have obtained the compounds that exert anti-HCV activity in the nanomolar range. Interestingly, these compounds did not inhibit the enzymatic activity of NS3 protease or NS5B RNA polymerase at the concentrations capable of inhibiting HCV replication in replicon cells.

2. Materials and methods

2.1. Compounds

More than 100 phenanthridinone derivatives were synthesized and used in this study. The synthesis of these compounds has been described previously [20,21]. Cyclosporin A (CsA) was purchased from Sigma–Aldrish. All compounds were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque) at a concentration of 20 mM or higher to exclude the cytotoxicity of DMSO and stored at −20 °C until use.

2.2. Cells

Huh-7 cells were grown and cultured in Dulbecco's modified Eagle medium with high glucose (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco/BRL), 100 U/ml penicillin G, and 100 μg/ml streptomycin. Huh-7 cells containing self-replicating subgenomic HCV replicons with a luciferase reporter, LucNeo#2 [22], were maintained in culture medium containing 1 mg/ml G418 (Nakarai Tesque). The subgenomic replicon cells without reporter #50-1 and the full-genomic replicon cells NNC#2 [23] were kindly provided by Dr. Hijikata (Kyoto University, Kyoto, Japan). These cells were also maintained in culture medium containing 1 mg/ml G418.

2.3. Anti-HCV assays

The anti-HCV activity of the test compounds was determined in LucNeo#2 cells by the previously described method with some modifications [24]. Briefly, the cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of various concentrations of the compounds. After incubation at 37 °C for 3 days, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Lysis buffer was added to each well, and the lysate was transferred to the corresponding well of a non-transparent 96-well plate. The luciferase activity was measured by addition of the luciferase reagent in a luciferase assay system kit (Promega) using a luminometer with automatic injectors (Berthold Technologies).

The activity of the test compounds was also determined by the inhibition of HCV RNA synthesis in LucNeo#2, #50–1, and NNC#2 cells [23,25]. The cells (5×10^3 cells/well) were cultured in a 96-well plate in the absence of G418 and in the presence of various concentrations of the compounds. After incubation at 37 °C for 3 days, the cells were washed with PBS, treated with lysis buffer in TaqMan® Gene Expression Cell-to-CT™ kit (Applied Biosystems), and the lysate was subjected to real-time reverse transcription polymerase chain reaction (RT-PCR), according to the manufacturer's instructions. The 5′-untranslated region of HCV RNA was quantified using the sense primer 5′-CGGGAGAGCCATAGTGG-3′, the antisense primer 5′-AGTACCACAAGGCCTTTCG-3′, and the fluorescence probe 5′-CTGCGGAACCGGTGAGTACAC-3′ (Applied Biosystems).

The inhibitory effect of the test compounds on the replication of a genotype 2a strain was evaluated by the infection of Huh-7.5.1 cells, kindly provided by Dr. Chisari at Scripps Institute, with cell-free JFH-1 virus, as previously described [11]. At 48 h after virus infection, the cells were treated with SideStep Lysis and Stabilization Buffer (Agilent Technologies), and the lysate was subjected to real-time RT-PCR for quantification of HCV RNA [25].

2.4. Cytotoxicity assay

Huh-7 cells (5×10^3 cells/well) were cultured in a 96-well plate in the presence of various concentrations of the test compounds. After incubation at 37 °C for 3 days, the number of viable cells was determined by a dye method using the water soluble tetrazolium Tetracolor One® (Seikagaku Corporation), according to the manufacturer's instructions. The cytotoxicity of the compounds was also evaluated by the inhibition of host cellular mRNA synthesis. The cells were treated with lysis buffer in the kit, as described above, and the cell lysate was subjected to real-time RT-PCR for amplification of a part of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA using a TaqMan® RNA control reagent (Applied Biosystems).

2.5. Immunoblotting

LucNeo#2 cells (5×10^3 cells/well) were cultured in a 96-well plate in the presence of various concentrations of the test compounds. After incubation at 37 °C for 4 days, the culture medium was removed, and the cells were washed with PBS and treated with lysis buffer (RIPA Buffer®, Funakoshi). The protein concentration of the lysate was measured by Bradford protein assay method (Bio-Rad). Then, the lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The primary antibodies used for protein detection were anti-NS3 (Thermo Scientific), anti-NS5A (Acris Antibodies), and anti-GAPDH (Santa Cruz Biotechnology) mouse monoclonal antibodies.

2.6. Protease and polymerase inhibition assays

The effect of the test compounds on NS3 protease activity was determined by a fluorescence resonance energy transfer-based assay using SensoLyte® 520 HCV Assay Kit (AnaSpec), according to the manufacturer's instructions. The inhibition assay for NS5B polymerase was performed at 37 °C for 60 min in a 384-well plate. A reaction mixture (30 μl/well) contains 20 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 0.05% Tween 20, 0.05% pluronic F127, 1 μM [^{3}H]GTP (0.1 μCi/well) plus cold GTP, 5 nM poly(rC), 62.5 nM biotinylated dG_{12}, 45 nM recombinant NS3 protease, and various concentrations of the compounds. The reaction was stopped by streatavidin scintillation proximity assay beads in 0.5 M ethylenediaminetetraacetic acid. The plate was counted with a microbeta reader on the following day.

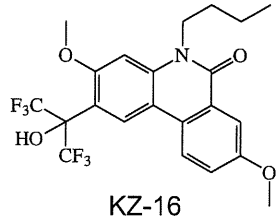
3. Results

When a number of phenanthridinone derivatives were examined for their antiviral activity in LucNeo#2 cells, three phenanthridinone derivatives, 5-butyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-3,8-dimethoxyphenanthridin-6(5*H*)-one (KZ-16), 4-butyl-11-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-[1,3]dioxolo[4,5-c]

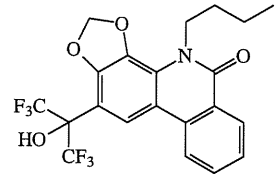
phenanthridin-5(4*H*)-one (HA-718), and 4-butyl-11-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-[1,3]dioxolo[4,5-*c*]phenanthridin-5(4*H*)-one (HA-718), and 4-butyl-11-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-7-methoxy-[1,3]dioxolo[4,5-*c*] phenanthridin-5(4*H*)-one (HA-719) (Fig. 1) proved to be highly potent and selective inhibitors of HCV (genotype 1b) replication. KZ-16, HA-718, and HA-719 reduced luciferase activity and viral RNA copy number in LucNeo#2 cells in a dose-dependent fashion (Fig. 2A–C). However, they did not affect the viability of Huh-7.5.1 cells at concentrations up to 40 μM (Fig. 2D). When the cytotoxicity of the compounds was evaluated by the copy number of GAPDH mRNA in the host cells, a similar result was obtained (data not shown).

Table 1 summarizes the anti-HCV activity of KZ-16, HA-718, and HA-719 in different (genotype 1b) replicon cells and in Huh-7 cells infected with cell-free JFH1 (genotype 2a) virus. The highest activity was achieved by HA-719 followed by KZ-16 and HA-718. The EC_{50} of KZ-16, HA-718, and HA-719 were 0.13 ± 0.04, 0.23 ± 0.06, and 0.063 ± 0.010 μM, respectively, in LucNeo#2 cells, when determined by the luciferase reporter activity. The 50% cytotoxic concentrations (CC_{50}) of all compounds were >40 μM. Therefore, the selectivity indices (SI), based on the ratio of CC_{50} to EC_{50}, of KZ-16, HA-718, and HA-719 were >307, >173, and >634, respectively. The anti-HCV activity of these compounds was confirmed by reduction of the viral RNA copy number in different replicon cells. However, they were less potent inhibitors of genotype 2a HCV (JFH1) replication in cell-free virus infection assay. Furthermore, the phenanthridinone derivatives were much less active in Huh-7 cells transfected with JFH1 replicons than in genotype 1b replicon cells (data not shown).

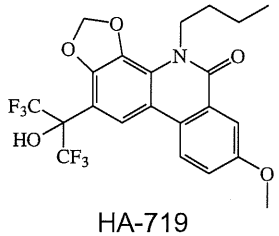
Immunoblot analysis was conducted to confirm that phenanthridinone derivatives were inhibitory to the expression of NS3 and NS5A proteins of HCV. As shown in Fig. 3, HA-719 strongly inhibited NS3 and NS5A expression in LucNeo#2 cells in a dose dependent fashion without affecting the expression of the host cellular protein GAPDH. The compound achieved 93% and 86% inhibition of NS3 and NS5A, respectively, at a concentration of 0.5 μM, indicating that HA-719 is a potent inhibitor of HCV protein expression as well as viral RNA synthesis. Immunoblot analysis was also conducted for another phenanthridinone derivative, 2-(2-benzyloxy-1,1,1,3,3,3-hexafluoropropan-2-yl)-5-butyl-3-methoxyphenanthridin-6(5*H*)-one (KZ-37), of which anti-HCV activity was weaker than HA-719. KZ-37 also proved inhibitory to NS3 and NS5A expression in a dose-dependent fashion (data not shown).



KZ-16



HA-718



HA-719

Fig. 1. Chemical structures of phenanthridinone derivatives.

In our attempt to elucidate the mechanism of action of the compounds, HA-719 was examined for their ability to inhibit the enzymatic activity of genotype 1b NS3 protease and NS5B polymerase in cell-free assay systems. Little, if any, inhibition of NS3 protease activity was observed for HA-719. Its 50% inhibitory concentration (IC_{50}) for the protease was 5.7 μM (data not shown), which was much higher than its EC_{50} for HCV replication in replicon cells (0.063–0.44 μM). HA-719 did not show any inhibitory effect on NS5B polymerase activity at concentrations up to 20 μM (data not shown). Furthermore, KZ-37, of which EC_{50} for HCV replication was 2.1–4.8 μM, was inactive against these two enzymes at a concentration of 20 μM (data not shown). Thus, it is unlikely that the phenanthridinone derivatives suppress HCV replication by inhibiting the activity of either NS3 protease or NS5B polymerase.

4. Discussion

In this study, we have demonstrated that novel phenanthridinone derivatives are potent and selective inhibitors of HCV replication in vitro. Our previous study on the synthesis and antiviral activity of phenanthridinone derivatives demonstrated that some of them exhibited selective but moderate activity against HCV replication in replicon cells [20,21]. After optimization of chemical structures, we succeeded in obtaining a series of potent and selective derivatives (Fig. 1). Among them, the most active one was HA-719, a novel phenanthridinone derivative with a dioxole structure.

Previous studies of HCV replicon cell systems indicated that most replicons had cell culture-adaptive mutations, which arose during the selection process with G418 and enhanced replication efficiency [26–29]. Self-replicating subgenomic RNA replicons could be eliminated from Huh-7 cells by prolonged treatment with IFN, and a higher frequency of cured cells could support the replication of subgenomic and full-genomic replicons [30]. The replication efficiency decreased with increasing amounts of transfected replicon RNA, indicating that viral RNA or proteins are cytopathic or that host cell factors in Huh-7 cells limit RNA amplification [31]. Therefore, both viral and cellular factors are considered to be important determinants for the efficiency of HCV replication in cell cultures, which may be able to explain the difference in EC_{50} values of the compounds among the subgenomic replicon cells used in this study (Table 1). Similarly, the difference in EC_{50} values in subgenomic and full-genomic replicon cells might be due to the difference of HCV RNA length or the difference of the host cells [32]. In fact, shorter RNA is known to replicate more efficiently than longer one [33].

The activity of phenanthridinone derivatives against the genotype 2a strain JFH1 was weaker than that against genotype 1b (Table 1). Although the assay systems were not the same (replicon cell assay for genotype 1b versus cell-free virus infection assay for genotype 2b), the compounds were much less active against genotype 2a (Table 1 and data not shown). Such difference in drug-sensitivity between genotype 1b and genotype 2a was previously reported and attributed to the genetic heterogeneity within the HCV genome [23]. In addition, the anti-HCV activity of compounds had been optimized in the genotype 1b replicon cells. HCV is classified into 6 genotypes that are further separated into a series of subtypes [34,35]. Among the genotypes, genotype 1b virus is epidemiologically predominant in Japan, and 65 and 17% of the cases

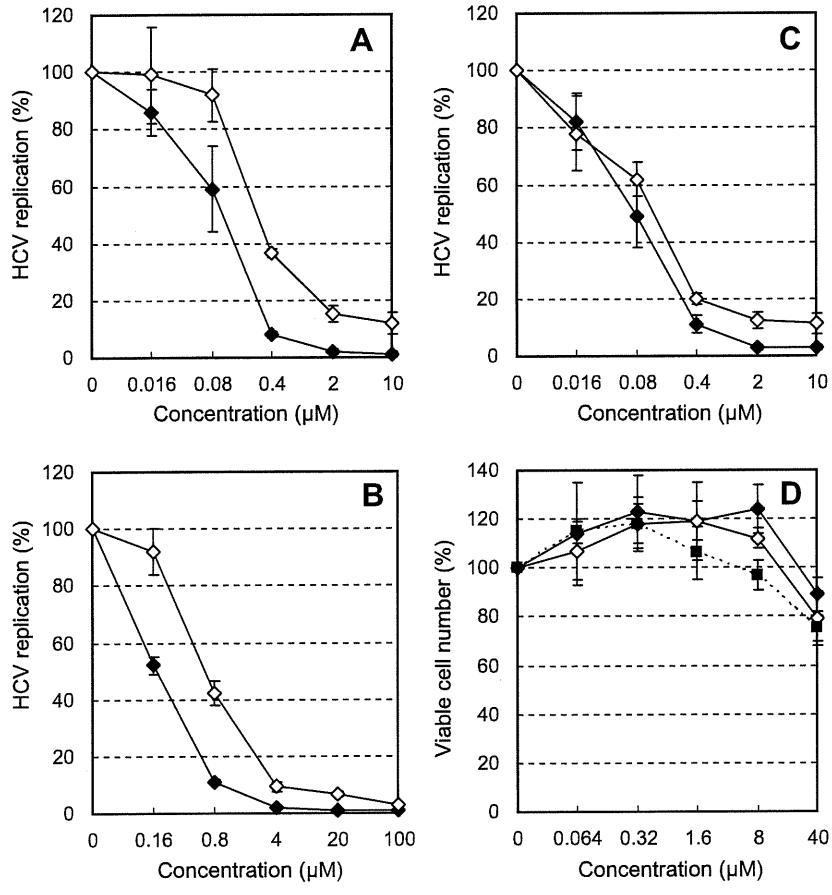


Fig. 2. Inhibitory effect of phenanthridinone derivatives on the replication HCV RNA replicons in LucNeo#2 cells and the proliferation of Huh-7 cells. LucNeo#2 cells were cultured in the presence of various concentrations of (A) KZ-16, (B) HA-718, or (C) HA-719. After incubation for 3 days, the cells were subjected to luciferase assay (closed diamond) and real-time RT-PCR (open diamond) to measure replicon-associated luciferase activity and RNA copy number, respectively, as parameters of HCV replication. (D) For the cell proliferation assay, Huh-7 cells were cultured in the presence of various concentrations of KZ-16 (closed diamond), HA-718 (open diamond), or HA-719 (closed square). After incubation for 3 days, the number of viable cells was determined by a tetrazolium dye method. Data represent means ± SD for triplicates experiments. Experiments were repeated at least twice, and a representative result is shown.

Table 1
Anti-HCV activity of phenanthridinone derivatives.

Compound		EC_{50} (μM)					CC_{50} (μM)
	Virus genotype	1b				2a	
	Cell	LucNeo#2		#50-1	NNC#2	Huh-7.5.1	Huh-7
	Assay	Luciferase	Real-time RT-PCR				Tetrazolium
KZ-16		0.13 ± 0.04	0.28 ± 0.01	0.40 ± 0.12	0.40 ± 0.14	2.6 ± 0.9	>40
HA-718		0.23 ± 0.06	0.68 ± 0.02	0.97 ± 0.56	0.90 ± 0.44	14 ± 5	>40
HA-719		0.063 ± 0.010	0.14 ± 0.01	0.25 ± 0.05	0.44 ± 0.20	4.9 ± 2.2	>40
CsA		0.24 ± 0.05	0.16 ± 0.01	0.18 ± 0.03	N.D.	0.58 ± 0.01	12 ± 3

EC_{50}: 50% effective concentration; CC_{50}: 50% cytotoxic concentration. N.D.: not determined.
Antiviral assay against the genotype 2a HCV was evaluated by the infection of Huh-7.5.1 cells with cell-free JFH-1 virus (see Section 2).
Except for the results in NNC#2 cells, all data represent means ± SD for three independent experiments. The data in NNC#2 cells represent means ± ranges for two independent experiments.

of HCV-related chronic hepatitis were caused by genotype 1b and genotype 2b, respectively [36].

At present, the target molecule of our phenanthridinone derivatives for inhibition of HCV replication remains unknown. Although it cannot be completely excluded that the compounds are inhibitors of NS3 protease or NS5B polymerase, biochemical assays revealed that HA-719 proved not to inhibit the activity of these enzymes at the concentrations capable of inhibiting viral replication. Therefore, the compounds may interact with another non-structural protein essential for viral replication, such as NS3 helicase and NS5A. In fact, a highly active inhibitor targeting NS5A has recently been identified [37]. Alternatively, the phenanthridinone derivatives may inhibit HCV replication through the interaction with host cellular factors deeply involved in HCV replication process [38–40]. It was reported that PJ34, a phenanthridinone derivative, had immunomodulatory activities and was protective against autoimmune diabetes [41], liver cancer [42], and stroke [43]. These studies suggested that the effects of PJ34 were attributed to the inhibition of poly(ADP-ribose) polymerase (PARP). Therefore, HA-719 was tested for its inhibitory effect on

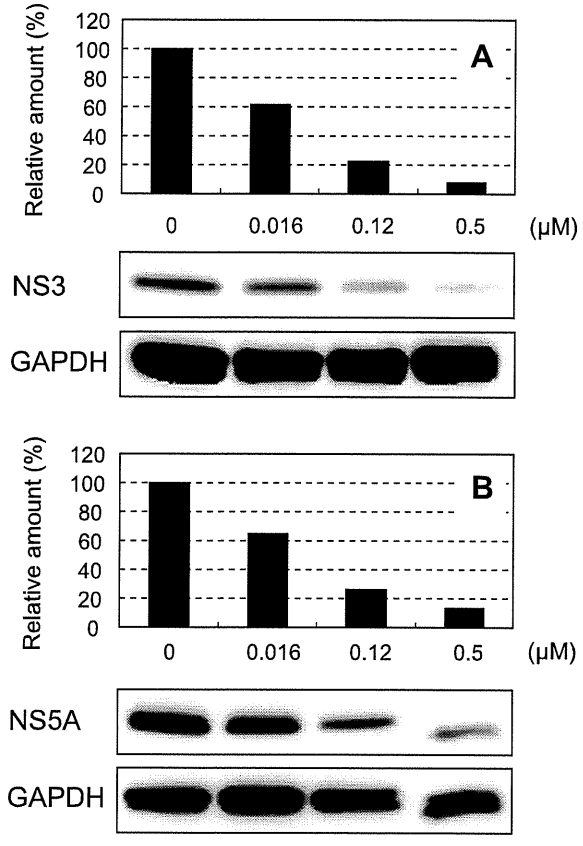


Fig. 3. Inhibitory effect of HA-719 on the expression of HCV proteins in LucNeo#2 cells. The cells were cultured in the presence of various concentrations of the compound. After incubation for 4 days, the cells were subjected to electrophoresis and immunoblot analysis for expression of (A) NS3 and (B) NS5A proteins. The band images were quantified by an image scanner and densitometer. Experiments were repeated at least twice, and a representative result is shown.

PARP activity and found to be inactive (data not shown). It was reported that some phenanthridinone derivatives had anti-human immunodeficiency virus (HIV) activity through the inhibition of viral integrase [44]. However, our compounds did not show selective inhibition of HIV replication in cell cultures (data not shown). Further studies, including the establishment of drug-resistant replicons, are in progress to determine the mechanism of action of the phenanthridinone derivatives.

In conclusion, our results clearly demonstrate that the novel phenanthridinone derivatives, especially HA-719, are highly potent and selective inhibitors of HCV replication in vitro. Although further studies, such as determination of their target molecule and pharmacological properties in vivo, are required, this class of compounds should be pursued for their clinical potential in the treatment of HCV infection.

Acknowledgments

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REVIEW

Changing etiologies and outcomes of acute liver failure: A perspective from Japan

Makoto Oketani, Akio Ido and Hirohito Tsubouchi

Department of Digestive and Life-Style Related Disease, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

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Correspondence
Dr. Makoto Oketani, Department of Digestive and Life-Style Related Disease, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. Email: oketani@m2.kufm.kagoshima-u.ac.jp

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Abstract

Acute liver failure in Japan usually consists of fulminant hepatitis (FH) due to viral infection, autoimmune hepatitis and drug-allergy-induced liver injury. The annual incidence of FH was estimated at 429 cases in 2004. FH is classified into acute or subacute type, and the prognosis of the latter is poor. Hepatitis B virus (HBV) is the most frequently identifiable agent that causes FH in Japan. Transient HBV infection is more prevalent in the acute than subacute type, whereas the frequency of HBV carriers is greater in the subacute type. FH due to HBV reactivation from resolved hepatitis B has been increasingly observed in patients with malignant lymphoma treated with rituximab and corticosteroid combination therapy. The prognosis is poor in HBV carriers with acute exacerbation, especially in patients with HBV reactivation from resolved hepatitis B. Despite careful investigation, the etiology is still unknown in 16% and 39% of the acute and subacute type of FH, respectively. Autoimmune hepatitis and drug-allergy-induced liver injury are found in 7% and 10%, respectively, and are more frequently observed in the subacute type of FH. Living donor liver transplantation is now the standard care for individuals with poor prognosis. Artificial liver support with plasmapheresis and hemodiafiltration plays a central role while waiting for a donor liver or for the native liver to regenerate. Further research is necessary to identify the causes of unknown origin. In addition, to improve the prognosis of FH, it is necessary to establish treatment modalities that are effective for liver regeneration.

Introduction

Acute liver failure is a clinical syndrome that is marked by the sudden loss of hepatic function in a person without chronic liver disease. The causes of acute hepatic failure are varied and differ geographically. In Japan, fulminant hepatitis (FH) is defined as having hepatitis, when grade II or worse hepatic encephalopathy develops within 8 weeks of the onset of the disease symptoms, with a prothrombin time of ≤40%. FH due to viral infection, autoimmune hepatitis and drug-allergy-induced liver injury is the main cause of acute liver failure in Japan. In contrast, other causes, including paracetamol overdose, other drug toxicity, metabolic liver disease, and acute fatty liver of pregnancy, are infrequent.

The Intractable Hepato-biliary Diseases Study Group of Japan annually performs a nationwide survey of patients with FH and late-onset hepatic failure (LOHF). This paper summarizes the results of the survey and addresses the characteristics and trends of acute liver failure in Japan.

Definition and methods

In 1969, Trey and Davidson defined acute liver failure as the occurrence of encephalopathy within 8 weeks of the onset of acute hepatic illness, and in the absence of pre-existing liver disease.[1] Thereafter, patients with hepatic encephalopathy that develops between 8 and 24 weeks after disease onset are defined as having LOHF.[2] Other definitions based on the duration of illness have subsequently been used to classify patients:[2–4] hyperacute, <7 days; acute, 7–28 days; and subacute, 28 days to 6 months. In Japan, patients with FH are classified into acute or subacute type, in which the encephalopathy occurs within 10 days, or later than 11 days, respectively, of the onset of disease symptoms.[5,6] Based on the previous survey, patients with FH who present within 10 days of symptom onset have significantly higher survival rates than similar patients who present with encephalopathy at 10 days after symptom onset.[7,8]

The survey was performed in hospital with active members of the Japan Society of Hepatology and the Japanese Society of Gastroenterology. Patients who meet the diagnostic criteria for FH and LOHF were entered into the survey (Table 1). Besides the diagnostic criteria, patients under 1 year of age and those with alcoholic hepatitis were excluded from the analysis.

The etiology of acute liver failure is classified into five categories: viral infection, autoimmune hepatitis, drug-allergy-induced liver injury, unknown, and indeterminate (Table 2). Patients with viral infection consist of those with hepatitis A virus (HAV),

Table 1 Diagnostic criteria for fulminant hepatitis in Japan according to the Intractable Liver Diseases Study Group of Japan, the Ministry of Health, Welfare and Labour (2003)

Fulminant hepatitis (FH) is defined as hepatitis in which hepatic encephalopathy of coma grade greater than II develops in the patients within 8 weeks after the onset of disease symptoms with highly deranged liver functions showing prothrombin time less than 40% of the standardized values.

FH is classified into two subtypes: the acute typeand subacute type in which the encephalopothy occurs within 10 days and later than 11 days, respecitvely.

Note 1: Patients with chronic liver diseases are excluded from FH, but asymptomatic HBV carriers who develop acute exacerbation are diagnosed with FH.

Note 2: Acute liver failure accompanying no liver inflammation, such as drug or chemical intoxication, microcirculatory disturbance, acute fatty liver of pregnancy, and Reye's syndrome are excluded from FH.

Note 3: The grading of hepatic encephalopathy is based on the criteria from the Inuyama Symposium in 1972.

Note 4: The etiology of FH is based on the criteria from the Intractable Liver Diseases Study Group of Japan in 2002 (Table 2).

Note 5: Patients with no hepatic encephalopathy or encephalopathy of coma grade I, even showing prothrombin time <40% of the standardized values, are diagnosed with severe acute hepatitis. Patients in whom encephalopathy develops between 8 and 24 weeks after disease onset, with prothrombin time <40% of the standardized values, are diagnosed with late onset hepatic failure (LOHF). Both are related to FH, but are regarded differently from FH.

Table 2 Criteria for etiology of fulminant hepatitis and late onset hepatic failure

I. Viral infection
  1. HAV: positive for serum IgM anti-HAV
  2. HBV: positive for either serum HBsAg, IgM anti-HBc or HBV DNA
    A. Transient infection: fulfilling either (a) or (b):
      (a) Negative for serum HBsAg before onset of acute liver injury.
      (b) Positive for serum IgM anti-HBc and negative for anti-HBc in serum diluted to 1:200.
    B. Acute exacerbation of carrier status: fulfilling either (a) or (b):
      (a) Positive for serum HBsAg before onset of acute liver injury
      (b) Negative for serum IgM anti-HBc and positive for anti-HBc in the serum diluted to 1:200.
    C. Undetermined: neither (a) nor (b)
  3. HCV: fulfilling either (a) or (b):
    (a) Negative for serum anti-HCV or HCV RNA before onset of acute liver injury.
    (b) Positive for serum HCV RNA and low titer positive for serum anti-HCV core protein.
  4. HEV: positive for serum HEV-RNA
  5. Other virus: e.g. EBV.

II. Autoimmune hepatitis: fulfilling either (a) (b) or (c):
  (a) Diagnosed as definite or probable according to the International Scoring System for autoimmune hepatitis.
  (b) Attenuation of liver injury after glucocorticosteroid administration and/or aggravation of liver injury following withdrawal of glucocorticoid.
  (c) Positive for serum antinuclear antigen and/or serum IgG levels >2 g/dL.

III. Drug-allergy-induced: drugs responsible for liver injury are determined by clinical course of liver injury and/or d-LST.

IV. Unknown: etiology is unknown despite sufficient examinations available.

V. Undetermined: etiology is undetermined because of insufficient examinations.

HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; EBV, Epstein-Barr virus; d-LST, drug-induced lymphocyte stimulation test.

hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis E virus (HEV) and other viruses. Patients with HBV infection are further classified into transient infection and acute exacerbation of HBV carrier status. In 2002, the criteria were modified to define FH due to autoimmune hepatitis and HEV, and the etiology of patients between 1998 and 2001 was re-assessed according to these new criteria.

Demographic features

From 1998 to 2006, 934 patients were enrolled in the surveillance.[9] Among these patients, 856 (432, acute type and 424, subacute type) were classified as having FH and 78 as having LOHF (Table 3). Based on the nationwide epidemiology surveillance, the annual incidence of FH was estimated at 3700 cases in 1972, 1050 cases in 1995, and 429 cases in 2004.[10] About 30% of patients with severe acute hepatitis were presumed to develop hepatic encephalopathy of coma grade II or more.[11]

The male : female ratio was higher for the acute type than subacute type and LOHF. The age of the patients was significantly higher for the subacute type and LOHF than for the acute type. The frequency of HBV carriers was highest for the subacute type and lowest for LOHF. There were many patients with complications, such as metabolic syndrome, malignancy and psychiatric disorders, which preceded the onset of acute liver failure, and most of these patients had received daily medication. This tendency was more obvious in patients with the subacute type and LOHF.

The survival rates of non-liver-transplanted patients were 54% for acute and 24% for subacute type FH, and 15% for LOHF. The prognosis of patients with subacute type FH and LOHF was evidently poor. These annual rates have not improved between 1998 and 2006. When compared to a previous survey,[12] prognosis of FH in acute type patients improved until 1998, although the prognosis remained poor in the subacute type with no liver transplantation during that period (Fig. 1). This improvement was probably achieved by progress in artificial liver support.

Causes of FH

Viral hepatitis

In Japan, the cause of FH has been identified as HAV, HBV or other viruses in about 50% of patients (Table 4). The causes of acute liver failure differed depending on the disease type. The frequencies of viral infection were 69% and 31% for patients with the acute and subacute types of FH, respectively, and 17% for LOHF patients.

Table 3 Demographic features of patients with fulminant hepatitis (FH) and late onset hepatic failure (LOHF) in Japan (1998–2006)

		FH		LOHF
	Total (n = 856)	Acute type (n = 432)	Subacute type (n = 424)	(n = 78)
Men/women	431/423	228/203	197/226	33/45
Age (years; mean ± SD)	48 ± 17	46 ± 16	49 ± 17**	53 ± 15**
HBV carrier rate (%)	14	12	16*	7***
Complications (%)	39	35	44*	49*
History of medication (%)	46	41	51**	54*
Survival rate (no LT) (%)	40	54	24**	15**
Survival rate (LT) (%)	77	73	79	81

*P < 0.05; **P < 0.01 versus acute type; ***P < 0.05 versus subacute type.
HBV, hepatitis B virus; LT, liver transplantation.

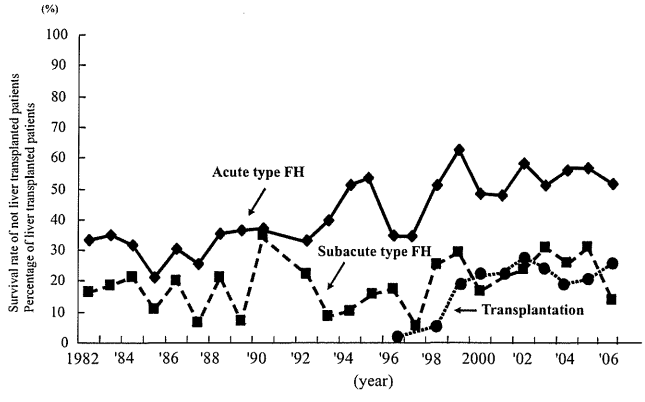

Figure 1 Survival rate of not liver transplanted patients with fulminant hepatitis(FH) and percentage of liver transplanted patients.

Infection with HAV was found in 6% of patients with FH and frequently observed in the acute type. As annual incidence of acute hepatitis A has declined over the past decade,[13] so too has the incidence of FH. However, as the overall immunity of the Japanese population to hepatitis A is only 12%[14] and is decreasing gradually as in other non-endemic areas, the increasing risk of future outbreaks of acute hepatitis A is probable. With regard to the severity of hepatitis A, age, sex, and drug toxicity have been identified as potential contributing factors.[15] HAV susceptibility and the risk of severity have likely increased recently.

In most of the patients, viral infections were due to HBV. HBV infection was found in 42% of patients with FH and 13% of those with LOHF. Among these, transient HBV infection was more frequent than acute exacerbation of HBV carrier status. Transient HBV infection was more frequent in the acute type (40%) than subacute type (9%) of FH, whereas the frequency of HBV carrier status was greater in the subacute type (16%) than in the acute type (11%). Annual incidence of FH due to HBV infection, both in transient HBV infection and acute exacerbation of HBV carrier status, has declined over the past decade. The routes of transmission of HBV indicate that, at present, sexual transmission from HBV carriers is a major route for FH. The preventive administration of HBV hyperimmune globulin and vaccination against HBV of neonates born to HBV-carrier mothers has been practiced nationwide since 1985 in Japan.[16] Therefore, the HBV carrier rate in the population has significantly decreased, and as a result, a marked decrease in the incidence of FH caused by HBV is expected.

Table 4 Percentage etiology of fulminant hepatitis (FH) and late onset hepatic failure (LOHF) in Japan (1998–2006)

		FH		LOHF
	Total (n = 856)	Acute type (n = 432)	Subacute type (n = 424)	(n = 78)
Viral infection	51	69	31	17
HAV	6	11	1	1
HBV	42	56	27	13
(Transient infection)	(25)	(40)	(9)	(5)
(Carrier)	(13)	(11)	(16)	(4)
(Undetermined)	(4)	(6)	(2)	(4)
HCV	1	1	1	1
HEV	1	1	1	0
Other virus	1	1	1	1
Autoimmune hepatitis	7	2	12	18
Drug-allergy-induced	10	8	13	15
Unknown	30	18	42	47
Indeterminate	3	3	3	3

HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Reactivation of HBV is a well-recognized complication in patients with chronic HBV infection who are undergoing cytotoxic chemotherapy or immunosuppressive therapy. HBV reactivation can be clinically severe and result in death from acute liver failure. Among acute exacerbation of HBV carrier status in the survey, HBV reactivation has been increasingly observed in patients with hematological malignancies. Furthermore, among the 12 patients with HBV reactivation, six with serological evidence of resolved hepatitis B [without hepatitis B surface antigen (HBsAg), but with antibody to hepatitis B core antigen (anti-HBc) and/or antibody to HBsAg (anti-HBs) in serum] developed reactivation with reappearance of HBsAg in serum. Most of these patients had received rituximab and coriticosteroid. Recently, combination therapy with rituximab and corticosteroid has been identified as a risk factor for HBV reactivation in HBsAg-negative patients with malignant lymphoma.[17,18] A study in Japan has revealed that 22% of *de novo* hepatitis B and that caused by HBV reactivation from resolved

hepatitis developed into fulminant hepatic failure, and mortality was 100%.[19] This problem deserves careful attention, because HBsAg-negative, anti-HBc-and/or anti-HBs-positive patients, which account for 20–25% of hospitalized patients in Japan, represent a high-risk group.[20]

HCV infection is rare in the etiology of patients with FH and LOHF. HCV infection was found in 1% of patients with FH, independent of the disease type. Reactivation of HCV as a cause of acute liver failure following chemotherapy has been reported.[21] However, none of these patients were found in the survey.

HEV infection was found in 1% of FH patients. HEV is a common cause of acute hepatitis in endemic areas, such as South Asia, Africa and South America.[22] The virus is now also known to exist indigenously in Japan, and can contribute to acute liver disease.[23,24] In Japan, the zoonotic transmission from pigs, wild boar and deer, either food-borne or otherwise, is the cause of HEV infection in non-endemic areas.[24,25] As for the geographical distribution of clinical HEV infection in Japan, it has been reported that there was wide variation with a higher prevalence in the northern part of Japan (Hokkaido Island and the northern part of mainland Honshu).[26] In the survey, two-thirds of the patients were from this area. Moreover, most of the patients were elderly men and there were no pregnant women, who have the highest attack rate of the virus in endemic areas.

In the survey, Epstein–Barr virus, cytomegalovirus, herpes simplex virus, human herpesvirus type-6 and parvovirus were infrequent causes of other forms of viral hepatitis.

Autoimmune hepatitis

Although autoimmune hepatitis is a chronic disease, an acute presentation occurs in approximately 22% of patients, and an even smaller number present with acute liver failure.[27] In the survey, autoimmune hepatitis was found in 7% of patients with FH and 18% of those with LOHF, respectively. In 2001, FH due to autoimmune hepatitis was recognized in Japan, because there were patients with non-HAV/HBV FH in which IgG levels were >2 g/dL, with positive antinuclear antigen in the serum. Although the diagnosis generally relies on the presence of serum autoantibodies, higher IgG levels (>2 g/dL), liver histology (if available), and response to corticosteroid therapy, the diagnosis of acute-onset autoimmune hepatitis is often difficult. The serum gammaglobulin or IgG concentrations are often lower than those in patients with chronic hepatitis.[28]

Drug-allergy-induced liver injury

Formation of toxic reactive metabolites has been suggested as a potential mechanism for causing idiosyncratic drug-induced liver injury.[29] Drug-allergy-induced liver injury was seen in 13% of patients with subacute type FH and in 15% of those with LOHF. The diagnosis relied mostly on the clinical course or drug-induced lymphocyte stimulation test (D-LST). Numerous types and classes of drugs have been implicated. Anti-tuberculosis agents (isoniazid, rifampicin, ethambutol and pyrazinamide), nonsteroidal anti-inflammatory drugs (loxoprofen, lornoxicam and acetaminophen), anti-cancer agents (tegafur, UFT and flutamide), drugs for metabolic syndrome (allopurinol and acarbose), and various herbal and natural remedies were the probable causative agents in the survey.

Table 5 Survival rates and etiology of patients with fulminant hepatitis (FH) and late onset hepatic failure (LOHF) in Japan (1998–2006)

		FH		LOHF
	Total ($n = 678$)	Acute type ($n = 369$)	Subacute type ($n = 309$)	($n = 62$)
Viral infection	45	55	23*	36*
HAV	74	77	40	100
HBV	39	50	18*	38
(Transient infection)	(51)	(56)	(32*)	(33)
(Carrier)	(22)	(35)	(13*)	(67)
(Undetermined)	(23)	(33)	(0)	(0)
HCV	67	75	60	0
HEV	60	100	33	—
Other virus	60	50	67	0
Autoimmune hepatitis	21	25	21	18
Drug allergy-induced	42	58	29*	0*
Unknown	36	54	26*	10*
Indeterminate	28	36	14	0

*$P < 0.05$ versus acute type.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Unknown etiology

The etiology was unknown in 42% and 47% of patients with subacute type FH and LOHF, respectively. Although the roles of GB virus C (GBV-C)/hepatitis G virus (HGV) and transfusion transmitted virus (TTV) have been discussed, in this survey, neither GBV-C/HGV or TTV appeared to be a major cause of FH. It is possible that the patients with drug-allergy-induced liver injury were contaminated with those of unknown etiology, because the ratio of medication history was high in these patients. The relationship between daily dose of oral medication or medication with significant hepatic metabolism and idiosyncratic drug-induced liver injury has been reported.[30,31] The higher numbers of patients with complications and daily medication in the survey support this evidence. Furthermore, HEV infection needs further investigation, because serum HEV RNA and IgM antibody to HEV were measured less in the survey.

Prognosis

The prognosis of patients with FH and LOHF differed depending on the etiology (Table 5). It was excellent in patients with HAV infection: the survival rate was 77% and 40% in patients with acute and subacute types of FH, respectively, and 100% in those with LOHF. In contrast, the prognosis was especially poor in HBV carriers who showed acute exacerbation. The survival rates of acute and subacute types of FH were 35% and 13%, respectively. It is noteworthy that none of the patients with HBV reactivation from resolved hepatitis B after rituximab and corticosteroid combination therapy survived. In contrast, the survival rate was 56% in acute type FH and 32% in subacute type in patients with transient HBV infection. The prognosis was poor in autoimmune hepatitis independent of disease type. Prognosis was also poor in patients

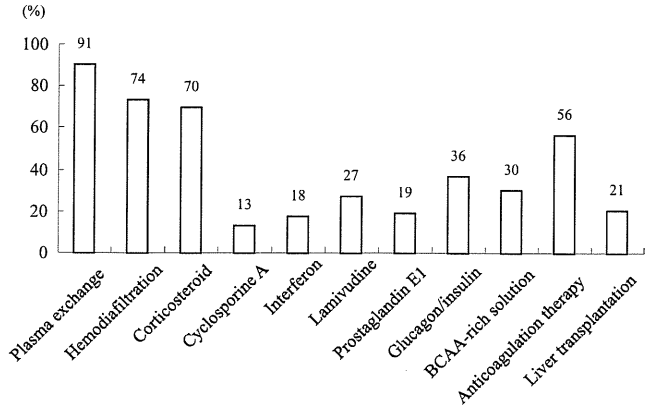

Figure 2 Percentage incidence of therapies performed for fulminant hepatitis (FH) and late onset hepatic failure (LOHF) in Japan (1998–2006). BCAA, branched-chain amino acid.

with subacute type FH and LOHF caused by drug-allergy-induced liver injury, and in those of the unknown etiology.

Complications

Complications that occurred during the course of acute liver failure also seemed to affect patient prognosis. Disseminated intravascular coagulation, renal failure and bacterial infection were found as complications in >30% of patients. Brain edema, gastrointestinal bleeding and congestive heart failure were seen in about 30%, 20% and 10%, respectively. Any of these complications significantly decreased survival rate. Furthermore, the number of these complications influenced prognosis.

Management

Specific therapies

The frequency of antiviral therapy with lamivudine has increased since 1998. As antiviral agents, lamivudine and interferon have been used in 27% and 18% of patients with FH and LOHF, respectively, between 1998 and 2006 (Fig. 2). Lamivudine has been used in 67% of patients with HBV-related FH or LOHF. Lamivudine has been reported to be efficacious for acute liver failure.[31,32] Recently, another guanosine nucleoside analog, entecavir, has been administered more frequently.[33] A preliminary study of entecavir for acute liver failure has revealed that the agent beneficially affects disease course. Lamivudine therapy is more efficacious when started early in acute liver failure. However, in the case of HBV reactivation from HBsAg-negative patients, it is difficult to prevent development of liver failure, even when lamivudine is administered after the onset of hepatitis. Two study groups in Japan have proposed guidelines for prevention of immunosuppressive-therapy- or chemotherapy-induced HBV reactivation. These guidelines recommend that patients with resolved infection should be routinely monitored for liver function and HBV DNA levels during and after chemotherapy, and antiviral therapy should be administered immediately when HBV DNA increases above the detection levels.

Corticosteroids were administered in 70% of patients with FH and LOHF. Steroid pulse therapy, methylprednisolone at a daily dose of 1 g injected intravenously, was administered to attenuate liver necrosis by suppressing excessive immune response. The efficacy of corticosteroids for improving the prognosis of acute liver failure is still obscure. Some randomized controlled trials have shown that corticosteroids provide no benefit overall in acute liver failure.[34] However, FH due to autoimmune hepatitis might be a candidate for therapy.[35] Anticoagulant therapy was performed in 56% of patients with FH and LOHF. Antithrombin III concentrate and protease inhibitor compounds such as gabexate mesylate and nafamostat mesylate were used as anticoagulants. They were effective for inhibition of disseminated intravascular coagulation and microcirculatory disturbance due to sinusoidal fibrin deposition. Glucagon/insulin, branched-chain amino acid-rich solution, cyclosporine A and prostaglandin E1 therapy was administered less frequently, and the frequency decreased compared to that in patients in the previous survey between 1995 to 1997.

Methods of liver support

In Japan, powerful artificial liver support with plasmapheresis and hemodiafiltration plays a central role in the treatment of acute liver failure. Plasmapheresis and hemodiafiltration were performed in 91% and 74% of patients with FH and LOHF, respectively (Fig. 2). In the late 1990s, hemodiafiltration therapy was developed and plasma exchange combined with hemodiafiltration therapy became popular. The increased frequency of this combination therapy in the 1990s could be implicated in the tendency for the survival rate to increase for acute type FH (Fig. 1). The effect of plasmapheresis on survival from acute liver failure has been difficult to determine. However, these support systems are efficacious for helping patients to remain in good condition until sufficient regeneration of the liver can be obtained, or liver transplantation can be performed. Recently, more powerful hemodiafiltration using large buffer volumes[36] or on-line hemodiafiltration[37] has been developed and has shown greater efficacy for improving hepatic coma.

Liver transplantation

Despite significant advances in critical care and an improved understanding of the pathophysiology of acute liver failure, the mortality rate remains high. Liver transplantation is the only life-saving treatment available beyond the supportive care of a critical unit. In Japan, living donors have been used because of the insufficiency of organ donation since 1988. Living donor liver transplantation was performed in 17% of patients with FH and LOHF between 1998 and 2006, and the frequency in those patients was significantly greater in the subacute type (21%) than in the acute type (13%). Recently, these frequency ratios have been almost steady (Fig. 1). The survival rates were 77% and 81% in patients with FH and LOHF, respectively, and there was no difference in the rates among the disease types. Patient and graft survival rates were 94% and 87% at 1 year, and 91% and 81% at 5 years, respectively. There was no significant difference in patient and graft survival according to etiology.[38]

Appropriate judgment to move forward to liver transplantation is the most important step. The indications for liver transplantation

in cases of FH are determined according to the 1996 Guidelines of the Acute Liver Failure Study Group of Japan. Re-evaluation of the guidelines has revealed that the accuracy in patients not receiving liver transplantation was 68% and 78% in acute and subacute types of FH, respectively, and 84% among those with LOHF.[39] The sensitivity and specificity of the assessment in patients with acute and subacute types were very low. To improve this situation, new guidelines for using a scoring system have been proposed by the Intractable Hepato-biliary Disease Study Group of Japan.[40] By using these guidelines, the accuracy in patients not receiving liver transplantation was increased to 75% and 87% in acute and subacute types of FH, respectively.

Experimental methods of liver support

To improve the prognosis of acute liver failure, advances in the treatment for liver regeneration are urgently needed. Hepatocyte growth factor (HGF) acts as a stimulator of liver regeneration, as well as an anti-apoptotic factor. We have started a clinical trial to examine the effects of recombinant human HGF (rhHGF) in patients with FHor LOHF, and in the four patients with FH or LOHF enrolled in this study; repeated doses of rh-HGF did not produce any severe side effects. Although two patients were rescued in this study, evaluation of this therapeutic agent is still under investigation.[41]

Several clinical trials of bone marrow cell infusion in patients with liver cirrhosis have shown clinical improvement. A clinical trial of autologous bone marrow infusion for patients with advanced liver cirrhosis due to chronic HBV infection has shown clinical improvement with no serious adverse events.[42] The recent discovery of pluripotent stem cells has yielded a new cell type for potential application in regenerative medicine. Strategies to achieve high levels of hepatocyte survival and the development of methods to engineer a functional liver system *in vivo* are expected in the future.

Conclusion

In Japan, the incidence of FH has decreased gradually and the clinical characteristics of patients and the therapeutic approach have changed in the past decade. The prognosis differs in patients with FH and LOHF depending on the disease type and etiology. HBV is the major cause of FH in Japan. Recently, careful attention has been necessary because of an increase in HBV reactivation from resolved hepatitis B. Despite careful investigation, a significant group with FH of unknown origin remains and needs further investigation. Living donor liver transplantation is the only life-saving treatment available beyond the supportive care of a critical unit. Artificial liver support systems are efficacious while waiting until the native liver regenerates or a donor is found. New therapeutic modalities are required to regenerate the liver, in particular, for the subacute type of FH.

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