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Endodermal and Hepatic Differentiation from Human Embryonic Stem Cells and Human Induced Pluripotent Stem Cells

Kenji Kawabata[1,2], Kazuo Takayama[1,3], Yasuto Nagamoto[1,3], Mary S. Saldon[1], Maiko Higuchi[1] and Hiroyuki Mizuguchi[1,3,4]*

[1]*Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan*
[2]*Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[3]*Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[4]*The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka 565-0871, Japan*

Abstract

Induced hepatocytes differentiated from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. Differentiation of human ESCs and iPSCs into endodermal and hepatic cell types has been achieved by several methods, including addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and a three-dimensional culture system. Each of these methods has an advantage from various points of view, such as the degree of maturation of differentiated hepatocytes, differentiation efficiency, clinical safety, and ease of handling. Currently, it is possible to select or combine the differentiation protocols to obtain ideal hepatocytes. The aim of this review is to describe the recent progress in endodermal and hepatic differentiation protocols from human ESCs and iPSCs in order to foster the suitable choice of induced hepatocytes on clinical and industrial applications.

Keywords: Embryonic stem cells; Induced pluripotent stem cells; Liver; Definitive endoderm; Differentiation

Introduction

The liver has many functions, including carbohydrate metabolism, glycogen storage, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins, and destruction of erythrocytes. The liver is composed of several types of cells, including epithelial, endothelial, and hematopoietic cells. Of these cells, hepatocytes play the most important role in major hepatic functions. Hepatocytes are thus useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly useful for drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models. For these applications, however, it is necessary to prepare a large number of the functional hepatocytes, which can no longer proliferate in *in vitro* culture. Isolated primary hepatocytes are the current standard *in vitro* model, because they express large amounts of drug-metabolizing enzymes and transporters [1]. However, isolated hepatocytes lose their differentiated properties, such as some cytochrome P450 activities that are induced by reference compounds, even under the optimized culture conditions [2,3]. Moreover, it can be difficult to set up long-term cultures with primary hepatocytes, because they can no longer proliferate in *in vitro* culture [4].

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body, and have the potential to provide an unlimited source of cells for a variety of applications [5-8]. Among the differentiated cells from ESCs and iPSCs, induced hepatocytes have a wide range of potential applications in biomedical research, drug discovery, and the treatment of liver disease. In this review, we provide an up-to-date overview of the wide variety of endodermal and hepatic differentiation protocols. These protocols were designed to reconstruct the *in vivo* environment in a variety of ways, including by addition of soluble factors into culture medium, transduction of differentiation-related genes, co-cultivation with other lineage cells, and use of a three-dimensional culture system.

Definitive Endoderm Differentiation from ESCs

Gastrulation of the vertebrate embryo starts with the formation of three germ layers: the ectoderm, mesoderm, and endoderm. The endoderm contributes to the digestive and respiratory tracts and their associated organs [9]. The endoderm differentiates into various organs, including the liver, pancreas, lungs, intestine, and stomach. To examine the molecular mechanisms of endoderm specification during early embryogenesis, endoderm differentiation from ESCs has been widely investigated as an *in vitro* model [10]. It has been reported that mouse ESCs have the ability to differentiate into definitive endoderm (DE) cells [11-13]. In recent studies, specific growth factors are used to generate DE cells from ESCs. In DE differentiation, it is well known that nodal signaling plays a crucial role and induces the expression of endoderm-related genes [14]. Activin A, a member of the nodal family, is a ligand of the type II activin receptor and can transmit a downstream signal by using Smad adaptor proteins [15-18]. Therefore, activin A is widely used to generate DE from ESCs. Although embryoid body (EB) formation is also used in the differentiation of ESCs, activin A could generate DE more efficiently than the EB formation [19]. In addition, using activin A with other factors such as fibroblast growth factor (FGF) 2 or Wnt3a proved to be more effective. Simultaneous addition of activin A and FGF2 could synergistically promote more efficient DE

***Corresponding author:** Dr. Hiroyuki Mizuguchi, Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel: +81-6-6879-8185; Fax: +81-6-6879-8186; E-mail: mizuguch@phs.osaka-u.ac.jp

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Research Article

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Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

Masayuki Kurosaki[1], Yasuhito Tanaka[2], Nao Nishida[3], Naoya Sakamoto[4], Nobuyuki Enomoto[5], Masao Honda[6], Masaya Sugiyama[2], Kentaro Matsuura[2], Fuminaka Sugauchi[2], Yasuhiro Asahina[1], Mina Nakagawa[4], Mamoru Watanabe[4], Minoru Sakamoto[5], Shinya Maekawa[5], Akito Sakai[6], Shuichi Kaneko[6], Kiyoaki Ito[7], Naohiko Masaki[7], Katsushi Tokunaga[3], Namiki Izumi[1,*], Masashi Mizokami[2,7]

[1]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan;* [2]*Department of Virology, Liver Unit, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan;* [3]*Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;* [4]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan;* [5]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan;* [6]*Department of Gastroenterology, Kanazawa University, Graduate School of Medicine, Kanazawa, Japan;* [7]*Research Center for Hepatitis and Immunology, International Medical Center of Japan, Konodai Hospital, Ichikawa, Japan*

Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, p <0.0001) and sustained virological response (SVR) (odds ratio = 7.41, p <0.0001) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.
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* Corresponding author. Address: Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. Tel.: +81 422 32 3111; fax: +81 422 32 9551.
E-mail address: nizumi@musashino.jrc.or.jp (N. Izumi).

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Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system

Kyoko Mori[a], Masanori Ikeda[a], Yasuo Ariumi[a], Hiromichi Dansako[a], Takaji Wakita[b], Nobuyuki Kato[a,*]

[a] Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Ribavirin (RBV) is a potential partner of interferon (IFN)-based therapy for patients with chronic hepatitis C. However, to date, its anti-hepatitis C virus (HCV) mechanism remains ambiguous due to the marginal activity of RBV on HCV RNA replication in HuH-7-derived cells, which are currently used as the only cell culture system for robust HCV replication. We investigated the anti-HCV activity of RBV using novel cell assay systems. The recently discovered human hepatoma cell line, Li23, which enables robust HCV replication, and the recently developed Li23-derived drug assay systems (ORL8 and ORL11), in which the genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase efficiently replicates, were used for this study. At clinically achievable concentrations, RBV unexpectedly inhibited HCV RNA replication in ORL8 and ORL11 systems, but not in OR6 (an HuH-7-derived assay system). The anti-HCV activity of RBV was almost cancelled by an inhibitor of equilibrative nucleoside transporters. The evaluation of the anti-HCV mechanisms of RBV proposed to date using ORL8 ruled out the possibility that RBV induces error catastrophe, the IFN-signaling pathway or oxidative stress. However, we found that the anti-HCV activity of RBV was efficiently cancelled with guanosine, and demonstrated that HCV RNA replication was notably suppressed in inosine monophosphate dehydrogenase (IMPDH)-knockdown cells, suggesting that the antiviral activity of RBV is mediated through the inhibition of IMPDH. In conclusion, we demonstrated for the first time that inhibition of IMPDH is a major antiviral target by which RBV at clinically achievable concentrations inhibits HCV RNA replication.

1. Introduction

Hepatitis C virus (HCV) infection causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma (Thomas, 2000). Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem (Thomas, 2000). HCV is an enveloped positive single-stranded RNA virus of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into in the following order: Core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato et al., 1990).

The current standard therapy for patients with chronic hepatitis C is a combination of pegylated-interferon (PEG-IFN) and ribavirin (RBV). This treatment currently achieves a sustained virological response (SVR) greater than 50% (Chevaliez et al., 2007). However, the mechanism of RBV activity in patients with chronic hepatitis C is still ambiguous. To date, five distinct mechanisms have been proposed: (a) RBV acts as an RNA mutagen that causes mutations of the HCV RNA genome and induces a so-called "error catastrophe" (Feld and Hoofnagle, 2005); (b) RBV enhances the IFN-signaling pathway (Feld et al., 2010; Thomas et al., 2011); (c) RBV induces GTP depression by inhibiting inosine monophosphate dehydrogenase (IMPDH) (Zhou et al., 2003); (d) RBV directly inhibits NS5B-encoded RNA-dependent RNA polymerase (Feld and Hoofnagle, 2005); (e) RBV enhances host T-cell mediated immunity by switching the T-cell phenotype from type 2 to type 1 (Lau et al., 2002). Unfortunately, no groups have clarified the anti-HCV mechanism of RBV at clinically achievable concentrations (5–14 μM) (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al.,

Abbreviations: HCV, hepatitis C virus; E1, envelope 1; NS2, nonstructural protein 2; PEG, polyethylene glycol; IFN, interferon; RBV, ribavirin; SVR, sustained virological response; IMPDH, inosine monophosphate dehydrogenase; RL, renilla luciferase; EC_{50}, 50% effective concentration; VE, vitamin E; NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine; MPA, mycophenolic acid; CsA, cyclosporine A; STAT1, signal transducer and activator of transcription 1; ENT, equilibrative nucleoside transporter; RT-PCR, reverse-transcription polymerase chain reaction; ISG, IFN-stimulated gene; IRF7, IFN regulatory factor 7; IP-10, IFN-gamma-inducible protein-10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5′-UTR, 5′-untranslated region; Neo^R, neomycin-resistance gene; CNT, concentrative nucleoside transporter; EC_{90}, 90% effective concentration; CFE, colony-forming efficiency; MMPD, merimepodib.

* Corresponding author. Tel.: +81 86 235 7385; fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).

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Stress-responsive maturation of Clk1/4 pre-mRNAs promotes phosphorylation of SR splicing factor

Kensuke Ninomiya, Naoyuki Kataoka, and Masatoshi Hagiwara

Department of Anatomy and Developmental Biology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

It has been assumed that premessenger ribonucleic acids (RNAs; pre-mRNAs) are spliced cotranscriptionally in the process of gene expression. However, in this paper, we report that splicing of *Clk1/4* mRNAs is suspended in tissues and cultured cells and that intermediate forms retaining specific introns are abundantly pooled in the nucleus. Administration of the Cdc2-like kinase–specific inhibitor TG003 increased the level of *Clk1/4* mature mRNAs by promoting splicing of the intron-retaining RNAs. Under stress conditions, splicing of general pre-mRNAs was inhibited by dephosphorylation of SR splicing factors, but exposure to stresses, such as heat shock and osmotic stress, promoted the maturation of *Clk1/4* mRNAs. Clk1/4 proteins translated after heat shock catalyzed rephosphorylation of SR proteins, especially SRSF4 and SRSF10. These findings suggest that *Clk1/4* expression induced by stress-responsive splicing serves to maintain the phosphorylation state of SR proteins.

Introduction

The appropriate phosphorylation of SR (serine–arginine-rich) proteins, a family of non–small nuclear RNP splicing factors, is essential for constitutive splicing and involved in regulation of alternative splicing (Duncan et al., 1997; Prasad et al., 1999; Lin and Fu, 2007; Long and Caceres, 2009), and both hypo- and hyperphosphorylations cause misregulation of splicing and splicing arrest. Phosphorylation states of SR proteins depend on the balanced activity between SR protein kinases and phosphatases. For example, heat shock–induced activation of PP1 (protein phosphatase 1) was reported to repress splicing through the dephosphorylation of SRSF10 (SRp38; Shin et al., 2004; Shi et al., 2006; Shi and Manley, 2007). SRPKs (SR protein kinases; Gui et al., 1994; Kuroyanagi et al., 1998; Wang et al., 1998), Cdc2-like kinases (Clks; Ben-David et al., 1991; Howell et al., 1991; Johnson and Smith, 1991; Nayler et al., 1997; Duncan et al., 1998), PRP4 (pre-mRNA processing 4; Alahari et al., 1993; Kojima et al., 2001), and dual-specificity tyrosine-regulated kinases (DYRKs; Alvarez et al., 2003; de Graaf et al., 2004) have been reported to phosphorylate SR proteins. To clarify the function of these kinases, we have developed synthetic inhibitors that specifically inhibit each target kinase (Hagiwara, 2005). We first found TG003, a kinase inhibitor specific for Clks (Muraki et al., 2004). TG003 affects splicing both in vitro and in vivo and suppresses influenza virus proliferation (Karlas et al., 2010; Nishida et al., 2011). We next synthesized SRPIN340, a specific inhibitor of SRPKs, which reduces the phosphorylation states of SR proteins (Fukuhara et al., 2006) and affects the splicing pattern of VEGF-A mRNA (Nowak et al., 2010). Recently, we reported on INDY1, a specific inhibitor of DYRKs, which rescues the transcriptional suppression of the NFAT1–regulated genes and abnormal development in *Xenopus laevis* embryo induced by DYRK overexpression (Ogawa et al., 2010).

The Clk family is a group of nuclear kinases for SR proteins and consists of four genes: ubiquitously expressed *Clk1*, *Clk2*, and *Clk4* and testis-specific *Clk3* (Nayler et al., 1997). Clks were demonstrated to be able to modulate splicing in vitro and in vivo (Colwill et al., 1996; Prasad et al., 1999; Yomoda et al., 2008). Clk1 and Clk4 are almost identical in amino acid sequence and considered to be functionally equivalent. After administration of TG003 to cultured cells, SR proteins, especially SRSF4 (SRp75), were dephosphorylated and accumulated in speckles within 60 min but rapidly rephosphorylated when the compound was washed out, indicating that constitutively active Clk1 or Clk4 is required to maintain the phosphorylation state of SR proteins (Yomoda et al., 2008).

Correspondence to Masatoshi Hagiwara: hagiwara.masatoshi.8c@kyoto-u.ac.jp

Abbreviations used in this paper: Clk, Cdc2-like kinase; dig, digoxigenin; DYRK, dual-specificity tyrosine-regulated kinase; NMD, nonsense-mediated mRNA decay.

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Chemical treatment enhances skipping of a mutated exon in the *dystrophin* gene

Atsushi Nishida[1,*], Naoyuki Kataoka[2,*], Yasuhiro Takeshima[1], Mariko Yagi[1], Hiroyuki Awano[1], Mitsunori Ota[1], Kyoko Itoh[3], Masatoshi Hagiwara[4,5] & Masafumi Matsuo[1]

Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease caused by a loss of the dystrophin protein. Control of dystrophin mRNA splicing to convert severe DMD to a milder phenotype is attracting much attention. Here we report a dystrophinopathy patient who has a point mutation in exon 31 of the *dystrophin* gene. Although the mutation generates a stop codon, a small amount of internally deleted, but functional, dystrophin protein is produced in the patient cells. An analysis of the mRNA reveals that the mutation promotes exon skipping and restores the open reading frame of dystrophin. Presumably, the mutation disrupts an exonic splicing enhancer and creates an exonic splicing silencer. Therefore, we searched for small chemicals that enhance exon skipping, and found that TG003 promotes the skipping of exon 31 in the endogenous *dystrophin* gene in a dose-dependent manner and increases the production of the dystrophin protein in the patient's cells.

[1] Department of Pediatrics, Kobe University Graduate School of Medicine, Chuo, 7-5-1 Kusunoki-cho, Kobe 650-0017, Japan. [2] Medical Top Track Program, Medical Research Institute, Tokyo Dental and Medical University, Tokyo 113-8510, Japan. [3] Department of Pathology and Applied Neurobiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan. [4] Department of Functional Genomics, Tokyo Dental and Medical University, Tokyo 113-8510, Japan. [5] Department of Anatomy and Developmental Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to M.H. (email: hagiwara.masatoshi.8c@kyoto-u.ac.jp) or to M.M. (email: matsuo@kobe-u.ac.jp).

Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

Naoya Sakamoto, MD, PhD,[1,2]* Mina Nakagawa,[1] Yasuhito Tanaka,[3] Yuko Sekine-Osajima,[1] Mayumi Ueyama,[1] Masayuki Kurosaki,[4] Nao Nishida,[5] Akihiro Tamori,[6] Nishimura-Sakurai Yuki,[1] Yasuhiro Itsui,[1,7] Seishin Azuma,[1] Sei Kakinuma,[1,2] Shuhei Hige,[8] Yoshito Itoh,[9] Eiji Tanaka,[10] Yoichi Hiasa,[11] Namiki Izumi,[4] Katsushi Tokunaga,[5] Masashi Mizokami,[12]Mamoru Watanabe[1] and the Ochanomizu-Liver Conference Study Group

[1]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
[2]*Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan*
[3]*Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku Nagoya, Japan*
[4]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[5]*Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[6]*Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, Japan*
[7]*Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan*
[8]*Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[9]*Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[10]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan*
[11]*Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan*
[12]*Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan*

Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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Naoya Sakamoto and Mina Nakagawa contributed equally to this work.

*Correspondence to: Naoya Sakamoto, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: nsakamoto.gast@tmd.ac.jp

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Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

Pattaratida Sa-nguanmoo[a,1], Yasuhito Tanaka[b,1], Parntep Ratanakorn[c], Masaya Sugiyama[d], Shuko Murakami[b], Sunchai Payungporn[e], Angkana Sommanustweechai[f], Masashi Mizokami[d], Yong Poovorawan[a,*]

[a] Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan
[c] Faculty of Veterinary Science, Mahidol University, Nakhon-Pathom 73170, Thailand
[d] The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Kounodai, Ichikawa 272-8516, Japan
[e] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[f] Dusit Zoo, Zoological Park Organization, Bangkok 10300, Thailand

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ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

* Corresponding author. Tel.: +66 2 2564909; fax: +66 2 2564929.
E-mail address: Yong.P@chula.ac.th (Y. Poovorawan).
[1] These authors contributed equally to this work.

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Dysfunction of Autophagy Participates in Vacuole Formation and Cell Death in Cells Replicating Hepatitis C Virus▽§

Shuhei Taguwa,[1]† Hiroto Kambara,[1]† Naonobu Fujita,[2] Takeshi Noda,[2] Tamotsu Yoshimori,[2] Kazuhiko Koike,[3] Kohji Moriishi,[4] and Yoshiharu Matsuura[1]*

Department of Molecular Virology, Research Institute for Microbial Diseases,[1] and Department of Genetics, Graduate School of Medicine,[2] Osaka University, Osaka 565-0871, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655,[3] and Department of Microbiology, Faculty of Medicine, Yamanashi University, Yamanashi 409-3898,[4] Japan

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Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma. Infection with the HCV genotype 1 in particular is considered a clinical risk factor for the development of hepatocellular carcinoma, although the molecular mechanisms of the pathogenesis are largely unknown. Autophagy is involved in the degradation of cellular organelles and the elimination of invasive microorganisms. In addition, disruption of autophagy often leads to several protein deposition diseases. Although recent reports suggest that HCV exploits the autophagy pathway for viral propagation, the biological significance of the autophagy to the life cycle of HCV is still uncertain. Here, we show that replication of HCV RNA induces autophagy to inhibit cell death. Cells harboring an HCV replicon RNA of genotype 1b strain Con1 but not of genotype 2a strain JFH1 exhibited an incomplete acidification of the autolysosome due to a lysosomal defect, leading to the enhanced secretion of immature cathepsin B. The suppression of autophagy in the Con1 HCV replicon cells induced severe cytoplasmic vacuolation and cell death. These results suggest that HCV harnesses autophagy to circumvent the harmful vacuole formation and to maintain a persistent infection. These findings reveal a unique survival strategy of HCV and provide new insights into the genotype-specific pathogenicity of HCV.

Hepatitis C virus (HCV) is a major causative agent of blood-borne hepatitis and currently infects at least 180 million people worldwide (58). The majority of individuals infected with HCV develop chronic hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma (25, 48). In addition, HCV infection is known to induce extrahepatic diseases such as type 2 diabetes and malignant lymphoma (20). It is believed that the frequency of development of these diseases varies among viral genotypes (14, 51). However, the precise mechanism of the genotype-dependent outcome of HCV-related diseases has not yet been elucidated. Despite HCV's status as a major public health problem, the current therapy with pegylated interferon and ribavirin is effective in only around 50% of patients with genotype 1, which is the most common genotype worldwide, and no effective vaccines for HCV are available (35, 52). Although recently approved protease inhibitors for HCV exhibited a potent antiviral efficacy in patients with genotype 1 (36, 43), the emergence of drug-resistant mutants is a growing problem (16). Therefore, it is important to clarify the life cycle and pathogenesis of HCV for the development of more potent remedies for chronic hepatitis C.

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita-shi, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.
† These authors contributed equally to this work.
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HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a single positive-stranded RNA genome with a nucleotide length of 9.6 kb, which encodes a single polyprotein consisting of approximately 3,000 amino acids (40). The precursor polyprotein is processed by host and viral proteases into structural and nonstructural (NS) proteins (34). Not only viral proteins but also several host factors are required for efficient replication of the HCV genome, where NS5A is known to recruit various host proteins and to form replication complexes with other NS proteins (39). In the HCV-propagating cell, host intracellular membranes are reconstructed for the viral niche known as the membranous web, where it is thought that progeny viral RNA and proteins are concentrated for efficient replication and are protected from defensive degradation, as are the host protease and nucleases (38).

Autophagy is a bulk degradation process, wherein portions of cytoplasm and organelles are enclosed by a unique membrane structure called an autophagosome, which subsequently fuses with the lysosome for degradation (37, 60). Autophagy occurs not only in order to recycle amino acids during starvation but also to clear away deteriorated proteins or organelles irrespective of nutritional stress. In fact, the deficiency of autophagy leads to the accumulation of disordered proteins that can ultimately cause a diverse range of diseases, including neurodegeneration and liver injury (12, 29, 30), and often to type 2 diabetes and malignant lymphoma (9, 32).

Recently, it has been shown that autophagy is provoked upon replication of several RNA viruses and is closely related to their propagation and/or pathogenesis. Coxsackievirus B3

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A new method for induced fit docking (GENIUS) and its application to virtual screening of novel HCV NS3-4A protease inhibitors.

Daisuke Takaya [a], Atsuya Yamashita [b], Kazue Kamijo [a], Junko Gomi [a], Masahiko Ito [b], Shinya Maekawa [c], Nobuyuki Enomoto [c], Naoya Sakamoto [d, e], Yoshiaki Watanabe [f], Ryoichi Arai [f], Hideaki Umeyama [f], Teruki Honma [a], Takehisa Matsumoto [a], Shigeyuki Yokoyama [*a, g].

[a] *RIKEN Systems and Structural Biology Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan.*
[b] *Department of Microbiology, Division of Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan*
[c] *First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan*
[d] *Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[e] *Department for Hepatitis Control, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[f] *School of Pharmacy, Kitasato University 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*
[g] *Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.*

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ABSTRACT

Hepatitis C virus (HCV) is an etiologic agent of chronic liver disease, and approximately 170 million people worldwide are infected with the virus. HCV NS3-4A serine protease is essential for the replication of this virus, and thus has been investigated as an attractive target for anti-HCV drugs. In this study, we developed our new induced-fit docking program (GENIUS), and applied it to the discovery of a new class of NS3-4A protease inhibitors. (IC50 =1~10μM including high selectivity index). The new inhibitors thus identified were modified, based on the docking models, and revealed preliminary structure-activity relationships. Moreover, the GENIUS *in silico* screening performance was validated by using an enrichment factor. We believe our designed scaffold could contribute to the improvement of HCV chemotherapy.

1. Introduction

Hepatitis C virus (HCV) is an etiologic agent of chronic liver disease[1,2], and approximately 170 million people worldwide are infected with the virus [3]. Chronic hepatitis C can lead to severe liver diseases, including fibrosis, cirrhosis and hepatocellular carcinoma [4]. The current standard therapy for chronic hepatitis C consists of pegylated interferon in combination with ribavirin [5]. Unfortunately, this therapy results in sustained antiviral activity in only about 50 to 60% of the patients, and is associated with serious side effects. Thus, the development of alternative and more effective anti-HCV agents has been eagerly anticipated.

HCV NS3-4A serine protease is essential for the replication of this virus, and has been investigated as an attractive target for anti-HCV drugs. Several three-dimensional structures of HCV NS3-4A protease have been deposited in the Protein Data Bank (PDB) [6]. Therefore, Structure Based Drug Design (SBDD) is a promising approach for the discovery of new NS3-4A protease inhibitors. The NS3-4A protease has the catalytic triad with the anion hole, commonly found among serine protease family members. The NS3-4A protease consists of two domains: a protease domain of 180 residues and a helicase domain of 420 residues [7]. The protease domain contains the protease activity, and thus it is appropriate to use only this domain as the receptor coordinates for SBDD [8]. On the other hand, docking calculations to a complex with a helicase domain have also been performed [9]. Different receptor structures were used in the docking calculations, because no experimentally determined full-length NS3-4A protease structures complexed with small molecule inhibitors were available, as of 2011.

In recent years, many peptide or peptide-mimic inhibitors that inhibit HCV NS3-4A protease have been developed, including SCH-503034[10], VX-950[11], BILN-2061[12], TMC-435[13], ITMN-191 [14] and MK-7009[15], as specifically targeted anti-viral agents for

* Corresponding author. Tel.: +0-000-000-0000; fax: +0-000-000-0000; e-mail: yokoyama@biochem.s.u-tokyo.ac.jp

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Review Article

Characterization of Kaposi's Sarcoma-Associated Herpesvirus-Related Lymphomas by DNA Microarray Analysis

Keiji Ueda, Eriko Ohsaki, Kazushi Nakano, and Xin Zheng

Division of Virology, Department of Microbiology and Immunology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

Correspondence should be addressed to Keiji Ueda, kueda@virus.med.osaka-u.ac.jp

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Among herpesviruses, γ-herpesviruses are supposed to have typical oncogenic activities. Two human γ-herpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), are putative etiologic agents for Burkitt lymphoma, nasopharyngeal carcinoma, and some cases of gastric cancers, and Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma (PEL) especially in AIDS setting for the latter case, respectively. Since such two viruses mentioned above are highly species specific, it has been quite difficult to prove their oncogenic activities in animal models. Nevertheless, the viral oncogenesis is epidemiologically and/or *in vitro* experimentally evident. This time, we investigated gene expression profiles of KSHV-oriented lymphoma cell lines, EBV-oriented lymphoma cell lines, and T-cell leukemia cell lines. Both KSHV and EBV cause a B-cell-originated lymphoma, but the gene expression profiles were typically classified. Furthermore, KSHV could govern gene expression profiles, although PELs are usually coinfected with KSHV and EBV.

1. Introduction

Several viruses could induce cancers in human beings. For examples, some papilloma viruses (PVs) should be etiologic agents for cervical cancers [1], hepatitis B virus (HBV) [2] and hepatitis C virus (HCV) [3] for hepatocellular carcinomas, human T-lymphotropic virus 1 (HTLV-1) for adult T-cell leukemia (ATL) [4], Epstein-Barr virus (EBV) for Burkitt lymphomas, nasopharyngeal carcinomas (NPCs), and some of gastric carcinomas [5, 6], and Kaposi's sarcoma-associated virus (KSHV) for Kaposi's sarcoma [7], primary effusion lymphomas (PELs), and multicentric Castleman's disease [8–13]. Recently, a newly identified polyomavirus, Merkel cell polyomavirus, is nominated as an etiologic agent for Merkel cell carcinoma [14]. These viruses have too narrow host ranges to meet Koch's principles, and, therefore, there are a lot of augments about it. Nevertheless, causation between the viral infection and the related cancer formation could be evident epidemiologically and *in vitro* experimentally.

Chronic inflammation caused by these viruses should be important factors, but it is not forgettable to keep in our minds that such inflammation itself is primarily caused by the viral infection [17]. Except for HCV and HTLV-1, these oncogenic viruses are usually DNA viruses and establish persistent or latent infection [18, 19]. Of course, HCV also establishes persistent infection in the infected hepatocytes [3]. Parts of some viral genomes in case of DNA viruses are integrated into host genomes, even though the process is not included in the life cycles. Integration could play roles for oncogenesis as shown for retroviral oncogenesis, and; thus, integration of viral genomes leads to promoter insertion mechanism to activate putative cellular oncogenes and host genome fragility [20]. If viral oncogenes are integrated and expressed, the effect should be more direct.

γ-herpesviruses such as EBV and KSHV are DNA viruses and do not have the genome integration process in their life cycles and just present as episomes in the infected nuclei for lives after establishing latent infection, since their genomes replicate and are partitioned according to the host cell cycles by utilizing host cellular replication machinery [6, 9]. Thus, the genomes act as complete extra genomes.

Efficient Generation of Functional Hepatocytes From Human Embryonic Stem Cells and Induced Pluripotent Stem Cells by HNF4α Transduction

Kazuo Takayama[1,2], Mitsuru Inamura[1,2], Kenji Kawabata[2,3], Kazufumi Katayama[1], Maiko Higuchi[2], Katsuhisa Tashiro[2], Aki Nonaka[2], Fuminori Sakurai[1], Takao Hayakawa[4,5], Miho Kusuda Furue[6,7] and Hiroyuki Mizuguchi[1,2,8]

[1]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [2]Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka, Japan; [3]Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [4]Pharmaceutics and Medical Devices Agency, Tokyo, Japan; [5]Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan; [6]JCRB Cell Bank, Division of Bioresources, National Institute of Biomedical Innovation, Osaka, Japan; [7]Laboratory of Cell Processing, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan; [8]The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan

Hepatocyte-like cells from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected to be a useful source of cells drug discovery. Although we recently reported that hepatic commitment is promoted by transduction of SOX17 and HEX into human ESC- and iPSC-derived cells, these hepatocyte-like cells were not sufficiently mature for drug screening. To promote hepatic maturation, we utilized transduction of the hepatocyte nuclear factor 4α (HNF4α) gene, which is known as a master regulator of liver-specific gene expression. Adenovirus vector-mediated overexpression of HNF4α in hepatoblasts induced by SOX17 and HEX transduction led to upregulation of epithelial and mature hepatic markers such as cytochrome P450 (CYP) enzymes, and promoted hepatic maturation by activating the mesenchymal-to-epithelial transition (MET). Thus HNF4α might play an important role in the hepatic differentiation from human ESC-derived hepatoblasts by activating the MET. Furthermore, the hepatocyte like-cells could catalyze the toxication of several compounds. Our method would be a valuable tool for the efficient generation of functional hepatocytes derived from human ESCs and iPSCs, and the hepatocyte-like cells could be used for predicting drug toxicity.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most of the body's cell types.[1,2] They could provide an unlimited source of cells for various applications. Hepatocyte-like cells, which are differentiated from human ESCs and iPSCs, would be useful for basic research, regenerative medicine, and drug discovery.[3] In particular, it is expected that hepatocyte-like cells will be utilized as a tool for cytotoxicity screening in the early phase of pharmaceutical development. To catalyze the toxication of several compounds, hepatocyte-like cells need to be mature enough to exhibit hepatic functions, including high activity levels of the cytochrome P450 (CYP) enzymes. Because the present technology for the generation of hepatocyte-like cells from human ESCs and iPSCs, which is expected to be utilized for drug discovery, is not refined enough for this application, it is necessary to improve the efficiency of hepatic differentiation. Although conventional methods such as growth factor-mediated hepatic differentiation are useful to recapitulate liver development, they lead to only a heterogeneous hepatocyte population.[4–6] Recently, we showed that transcription factors are transiently transduced to promote hepatic differentiation in addition to the conventional differentiation method which uses only growth factors.[7] Ectopic expression of Sry-related HMG box 17 (SOX17) or hematopoietically expressed homeobox (HEX) by adenovirus (Ad) vectors in human ESC-derived mesendoderm or definitive endoderm (DE) cells markedly enhances the endoderm differentiation or hepatic commitment, respectively.[7,8] However, further hepatic maturation is required for drug screening.

The transcription factor hepatocyte nuclear factor 4α (HNF4α) is initially expressed in the developing hepatic diverticulum on E8.75,[9,10] and its expression is elevated as the liver develops. A previous loss-of-function study showed that HNF4α plays a critical role in liver development; conditional deletion of *HNF4α* in fetal hepatocytes results in the faint expression of many mature hepatic enzymes and the impairment of normal liver morphology.[11] The genome-scale chromatin immunoprecipitation assay showed that HNF4α binds to the promoters of nearly half of the genes expressed in the mouse liver,[12] including cell adhesion and junctional proteins,[13] which are important in

Correspondence: *Hiroyuki Mizuguchi, Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: mizuguch@phs.osaka-u.ac.jp*

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Efficient and Directive Generation of Two Distinct Endoderm Lineages from Human ESCs and iPSCs by Differentiation Stage-Specific SOX17 Transduction

Kazuo Takayama[1,2], Mitsuru Inamura[1,2], Kenji Kawabata[2,3], Katsuhisa Tashiro[2], Kazufumi Katayama[1], Fuminori Sakurai[1], Takao Hayakawa[4,5], Miho Kusuda Furue[6,7], Hiroyuki Mizuguchi[1,2,8]*

1 Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan, **2** Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan, **3** Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan, **4** Pharmaceutics and Medical Devices Agency, Chiyoda-ku, Tokyo, Japan, **5** Pharmaceutical Research and Technology Institute, Kinki University, Higashiosaka, Osaka, Japan, **6** JCRB Cell Bank, Division of Bioresources, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan, **7** Laboratory of Cell Processing, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan, **8** The Center for Advanced Medical Engineering and Informatics, Osaka University, Suita, Osaka, Japan

Abstract

The establishment of methods for directive differentiation from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is important for regenerative medicine. Although Sry-related HMG box 17 (SOX17) overexpression in ESCs leads to differentiation of either extraembryonic or definitive endoderm cells, respectively, the mechanism of these distinct results remains unknown. Therefore, we utilized a transient adenovirus vector-mediated overexpression system to mimic the SOX17 expression pattern of embryogenesis. The number of alpha-fetoprotein-positive extraembryonic endoderm (ExEn) cells was increased by transient SOX17 transduction in human ESC- and iPSC-derived primitive endoderm cells. In contrast, the number of hematopoietically expressed homeobox (HEX)-positive definitive endoderm (DE) cells, which correspond to the anterior DE *in vivo*, was increased by transient adenovirus vector-mediated SOX17 expression in human ESC- and iPSC-derived mesendoderm cells. Moreover, hepatocyte-like cells were efficiently generated by sequential transduction of SOX17 and HEX. Our findings show that a stage-specific transduction of SOX17 in the primitive endoderm or mesendoderm promotes directive ExEn or DE differentiation by SOX17 transduction, respectively.

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* E-mail: mizuguch@phs.osaka-u.ac.jp

Introduction

There are two distinct endoderm lineages in early embryogenesis, the extraembryonic endoderm (ExEn) and the definitive endoderm (DE). The first of these lineages, the ExEn plays crucial roles in mammalian development, although it does not contribute to the formation of body cells. In early embryogenesis, a part of the inner cell mass of the blastocyst differentiates into the primitive endoderm (PrE). The PrE differentiates into the ExEn that composes the parietal endoderm, which contributes to the primary yolk sac, and the visceral endoderm, which overlies the epiblast [1,2]. In contrast, the second of the endoderm lineages, the DE arises from the primitive streak (PS), which is called the mesendoderm [3]. The DE has the ability to differentiate into the hepatic and pancreatic tissue [4].

The establishment of human embryonic stem cells (ESCs) [5] and human induced pluripotent stem cells (iPSCs) [6,7] has opened up new opportunities for basic research and regenerative medicine. To exploit the potential of human ESCs and iPSCs, it is necessary to understand the mechanisms of their differentiation. Although growth factor-mediated ExEn or DE differentiation is widely performed, it leads to a heterogeneous population [8,9,10,11]. Several studies have utilized not only growth factors but also modulation of transcription factors to control downstream signaling cascades [10,12,13]. Sox17, an Sry-related HMG box transcription factor, is required for development of both the ExEn and DE. In mice, during ExEn and DE development, Sox17 expression is first observed in the PrE and in the anterior PS, respectively [14]. Previous study showed that stable Sox17 overexpression promotes ExEn differentiation from mouse ESCs [12]. On the other hand, another previous study has demonstrated that DE progenitors can be established from human ESCs by stable expression of SOX17 [10]. The mechanism of these discrepancies which occurs in SOX17 transduction still remains unknown. Also, the role of SOX17 in human ExEn differentiation still remains unknown. Therefore, it is quite difficult to promote directive differentiation into either ExEn or DE cells by SOX17 transduction.

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Genome-wide association study identified *ITPA/DDRGK1* variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C

Yasuhito Tanaka[1,2,†], Masayuki Kurosaki[3,†], Nao Nishida[4], Masaya Sugiyama[1,2,5], Kentaro Matsuura[1,6], Naoya Sakamoto[7], Nobuyuki Enomoto[8], Hiroshi Yatsuhashi[9], Shuhei Nishiguchi[10], Keisuke Hino[11], Shuhei Hige[12], Yoshito Itoh[13], Eiji Tanaka[14], Satoshi Mochida[15], Masao Honda[16], Yoichi Hiasa[17], Asako Koike[18], Fuminaka Sugauchi[1,6], Shuichi Kaneko[16], Namiki Izumi[3], Katsushi Tokunaga[4] and Masashi Mizokami[5,*]

[1]Department of Virology and [2]Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, [3]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan, [4]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, [5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba, Japan, [6]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, [7]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan, [8]First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan, [9]Clinical Research Center, National Nagasaki Medical Center, Nagasaki, Japan, [10]Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan, [11]Division of Hepatology and Pancreatology, Kawasaki Medical College, Kurashiki, Japan, [12]Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan, [13]Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan, [14]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, [15]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Saitama Medical University, Saitama, Japan, [16]Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan, [17]Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan, and [18]Central Research Laboratory, Hitachi Ltd, Kokubunji, Japan

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Hematologic abnormalities during current therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) for chronic hepatitis C (CHC) often necessitate dose reduction and premature withdrawal from therapy. The aim of this study was to identify host factors associated with IFN-induced thrombocytopenia by genome-wide association study (GWAS). In the GWAS stage using 900K single-nucleotide polymorphism (SNP) microarrays, 303 Japanese CHC patients treated with PEG-IFN/RBV therapy were genotyped. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the minor-allele-dominant model with the decrease of platelet counts in response to PEG-IFN/RBV therapy [$P = 8.17 \times 10^{-9}$; odds ratio (OR) = 4.6]. These associations were replicated in another sample set ($n = 391$) and the combined *P*-values reached 5.29×10^{-17} (OR = 4.5). Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that rs11697186 at the GWAS stage had a strong linkage disequilibrium with rs1127354, known as a functional variant in the *ITPA* gene. The

*To whom correspondence should be addressed at: The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa, Chiba 272-8516, Japan. Tel: +81 473723501; Fax: +81 473754766; Email: mmizokami@hospk.ncgm.go.jp
†These authors contributed equally.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells

Shigeyuki Takeshita · Tatsuki Ichikawa · Naota Taura · Hisamitsu Miyaaki · Toshihisa Matsuzaki · Masashi Otani · Toru Muraoka · Motohisa Akiyama · Satoshi Miuma · Eisuke Ozawa · Masanori Ikeda · Nobuyuki Kato · Hajime Isomoto · Fuminao Takeshima · Kazuhiko Nakao

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Abstract
Background Geranylgeranylacetone (GGA), an isoprenoid compound which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating ISGF3 formation in human hepatoma cells. This drug has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.
Methods OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. After treatment, the cells were harvested with Renilla lysis reagent and then subjected to a luciferase assay according to the manufacturer's protocol.
Result The results showed that GGA had anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1, and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.
Conclusions GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

Keywords mTOR · STAT-1 · Interferon · HCV · GGA

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
GGA	Geranylgeranylacetone
siRNA	Small interfering RNA

S. Takeshita · T. Ichikawa (✉) · N. Taura · H. Miyaaki · T. Matsuzaki · M. Otani · T. Muraoka · M. Akiyama · S. Miuma · E. Ozawa · H. Isomoto · F. Takeshima · K. Nakao
Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: ichikawa@net.nagasaki-u.ac.jp

M. Ikeda · N. Kato
Department of Molecular Biology, Graduate school of Medicine and Dentistry, Okayama University, Okayama, Japan

Introduction

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are

Association between lipid accumulation and the cannabinoid system in Huh7 cells expressing HCV genes

MAKO TOYODA[1], AKIRA KITAOKA[1], KAZUYUKI MACHIDA[1], TAKUYA NISHINAKAGAWA[1], RYOKO YADA[3], MOTOYUKI KOHJIMA[4], MASAKI KATO[5], KAZUHIRO KOTOH[5], NAOYA SAKAMOTO[6], GOSHI SHIOTA[7], MAKOTO NAKAMUTA[3,4], MANABU NAKASHIMA[1] and MUNECHIKA ENJOJI[1,2,3]

[1]Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences and [2]Health Care Center Clinic, Fukuoka University, Fukuoka; [3]Clinical Research Center and [4]Department of Gastroenterology, Kyushu Medical Center, National Hospital Organization, Fukuoka; [5]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [6]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo; [7]Division of Molecular and Genetic Medicine, Department of Genetic Medicine and Regenerative Therapeutics, Graduate School of Medicine, Tottori University, Yonago, Japan

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Abstract. Evidence from clinical and laboratory studies has accumulated indicating that the activation of the cannabinoid system is crucial for steatosis, especially in non-alcoholic fatty liver disease. However, the association between hepatitis C virus (HCV) infection and the cannabinoid system has not been well investigated and it is unclear whether steatosis in chronic hepatitis C develops via activation of the endocannabinoid/cannabinoid receptor signaling pathway. In this study, we examined the expression of a cannabinoid receptor (CB1) and the lipid accumulation in the hepatic Huh7 cell line, expressing HCV genes. We utilized Huh7/Rep-Feo-1b cells stably expressing HCV non-structural proteins (NS) 3, NS4, NS5A, and NS5B, as well as Tet-On Core-2 cells, in which the HCV core protein expression is inducible. Significantly higher levels of stored triglycerides were found in Huh7/Rep-Feo-1b cells compared to Huh7 cells. Also, triglyceride accumulation and CB1 receptor expression were down-regulated in Huh7/Rep-Feo-1b cells after HCV reduction by IFNα. Moreover, lipid accumulation appeared to increase after CB1 agonist treatment, while it decreased after CB1 antagonist treatment, although significant differences were not found compared to untreated cells. In Tet-On Core-2 cells, induction of HCV core protein expression did not affect CB1 expression or triglyceride accumulation. The results of this study in cultured cells suggest that HCV infection may activate the cannabinoid system and precede steatosis, but the core protein by itself may not have any effect on the cannabinoid system.

Correspondence to: Dr Munechika Enjoji, Health Care Center, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
E-mail: enjoji@adm.fukuoka-u.ac.jp

Abbreviations: 2-AG, 2-arachidonoylglycerol; CH-C, chronic hepatitis C; FAAH, fatty-acid amide hydrolase; FASN, fatty acid synthase; MTTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBS, phosphate buffered saline; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element-binding protein 1c; VLDL, very low-density lipoprotein

Key words: cannabinoid, cannabinoid receptor 1, hepatitis C virus, triglyceride, Huh7, replicon

Introduction

Cannabinoids are hydrophobic fatty-acid-derived compounds with predominantly autocrine/paracrine effects acting via specific G-protein-coupled receptors (CB1 and CB2). Endocannabinoids, such as anandamide and 2-arachidonoylglycerol (2-AG), are not stored in cells and are synthesized on demand from lipid precursors in cellular membranes (1). They are released in response to specific stimuli and are rapidly degraded by fatty-acid amide hydrolase (FAAH) or monoacylglycerol lipase, following ligand binding and cellular uptake (2). Emerging evidence suggests the crucial role of the hepatic cannabinoid system, which appears to be mediated via activation of cannabinoid receptors in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (1-5). CB1 receptor stimulation contributes to liver steatosis progression and exerts profibrogenic and proinflammatory effects in the liver. Steatogenic agents such as a high-fat diet can up-regulate the activity of CB1 receptors via increasing the synthesis of endocannabinoids. In the NASH experimental model, hepatic anandamide levels increase following inhibition of its degradation by FAAH, and CB1 receptor expression is strongly increased (6). CB1 receptors contribute to metabolic steatosis and the related insulin resistance, and can also be up-regulated by obesity (5-7). CB1 receptor activation results

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Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents

Youki Ueda, Kyoko Mori, Yasuo Ariumi, Masanori Ikeda, Nobuyuki Kato *

Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a global health problem. HuH-7 hepatoma-derived cells are widely used as the only cell-based HCV replication system for HCV research, including drug assays. Recently, using different hepatoma Li23-derived cells, we developed an HCV drug assay system (ORL8), in which the genome-length HCV RNA (O strain of genotype 1b) encoding renilla luciferase replicates efficiently. In this study, using the HuH-7-derived OR6 assay system that we developed previously and the ORL8 assay system, we evaluated 26 anti-HCV reagents, which other groups had reported as anti-HCV candidates using HuH-7-derived assay systems other than OR6. The results revealed that more than half of the reagents showed different anti-HCV activities from those in the previous studies, and that anti-HCV activities evaluated by the OR6 and ORL8 assays were also frequently different. In further evaluation using the HuH-7-derived AH1R assay system, which was developed using the AH1 strain of genotype 1b, several reagents showed different anti-HCV activities in comparison with those evaluated by the OR6 and ORL8 assays. These results suggest that the different activities of anti-HCV reagents are caused by the differences in cell lines or HCV strains used for the development of assay systems. Therefore, we conclude that plural HCV assay systems developed using different cell lines or HCV strains are required for the objective evaluation of anti-HCV reagents.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma. Since approximately 170 million people are infected with HCV worldwide, HCV infection is a serious global health problem [1]. Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virologic response [2]. HCV is an enveloped virus with a positive single-stranded RNA virus of the *Flaviviridae* family. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3,4].

To date, HuH-7 hepatoma-derived cells are used as the only cell culture system for robust HCV replication in HCV research, including drug assays. We have also developed a HuH-7-derived drug assay system (OR6), in which genome-length HCV RNA (O strain of genotype 1b derived from an HCV-positive blood donor) encoding renilla luciferase (RL) efficiently replicates [5]. Recently, we found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication [6], and we showed that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells, although both cell lines had similar liver-specific expression profiles [7]. In that study, we identified three genes (New York esophageal squamous cell carcinoma 1, β-defensin-1, and galectin-3) showing Li23-specific expression profiles by a comparative analysis using several other hepatic cell lines [7]. We further developed Li23-derived drug assay systems (ORL8 and ORL11), which are relevant to the HuH-7-derived OR6 assay system [6]. During the process of evaluating the ORL8 and ORL11 assay systems using anti-HCV reagents such as IFNs, we noticed that these assay systems were frequently more sensitive to anti-HCV reagents than the OR6 assay system [6]. Furthermore, we recently found that ribavirin at clinically achievable concentrations (approximately 10 μM) effectively inhibited HCV RNA replication in both the ORL8 and ORL11 assay systems, but not in the OR6 assay system [8]. This finding led to the clarification of the anti-HCV mechanism of ribavirin, and we demonstrated that ribavirin's anti-HCV activity was mediated by the inhibition of inosine monophosphate dehydrogenase, a key enzyme in the guanosine biosynthetic pathway [8]. From these findings, we supposed that the anti-HCV reagents reported to date might show different activities among the different drug assay systems. To test this assumption, we evaluated 22 anti-HCV reagents that were reported using HuH-7-derived assay systems other than OR6, using the OR6 and ORL8 assay systems. Four additional

* Corresponding author. Fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).

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Original article

Serum interleukin-6 levels correlate with resistance to treatment of chronic hepatitis C infection with pegylated-interferon-α2b plus ribavirin

Mayumi Ueyama[1†], Mina Nakagawa[1,2†], Naoya Sakamoto[1,2], Izumi Onozuka[1], Yusuke Funaoka[1], Takako Watanabe[1], Sayuri Nitta[1], Kei Kiyohashi[1], Akiko Kitazume[1], Miyako Murakawa[1], Yuki Nishimura-Sakurai[1], Yuko Sekine-Osajima[1], Yasuhiro Itsui[3], Seishin Azuma[1], Sei Kakinuma[1,2], Mamoru Watanabe[1], the Ochanomizu-Liver Conference Study Group[‡]*

[1]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan
[3]Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan

*Corresponding author e-mail: nsakamoto.gast@tmd.ac.jp
[†]These authors contributed equally to this work
[‡]A list of the participating hospitals in the Ochanomizu-Liver Conference Study Group can be found in Additional file 1

Background: Interleukin (IL)-6, a pleiotropic cytokine, is increased in various types of chronic liver disease, including chronic hepatitis C (CHC). It was reported recently that IL-6 is associated with insulin resistance, iron metabolism and interferon resistance, which may affect the outcome of antiviral treatment. In this study, we investigated the association of serum IL-6 levels with outcomes of pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy.

Methods: We included 149 CHC patients and measured serum IL-6 levels at baseline and at 4, 8 and 12 weeks, and the end of treatment in 49 patients. We performed univariate and multivariate regression analyses for the association of IL-6 levels and clinical and laboratory parameters and treatment responses.

Results: Serum IL-6 levels were significantly higher in CHC patients than healthy subjects. Pretreatment IL-6 levels of male patients were inversely correlated with sustained virological response (SVR) in univariate analysis (P=0.012). In male patients with SVR, serum IL-6 levels decreased significantly at 4 weeks of treatment (P=0.029) and remained significantly lower than those of non-SVR patients after 4, 8 and 12 weeks of PEG-IFN plus RBV therapy.

Conclusions: Our results suggest that baseline levels of IL-6, as well as their decrease during treatment, are correlated to outcomes of PEG-IFN plus RBV therapy in male patients. Further analyses of IL-6 may provide new strategies for difficult-to-treat CHC patients and prevention of hepatocarcinogenesis.

Introduction

HCV is one of the main causes of chronic liver disease. Chronic hepatitis can result in hepatic fibrosis, liver cirrhosis [1] and hepatocellular carcinoma (HCC) [2,3]. The current standard of care consists of a combination of pegylated interferon (PEG-IFN)-α plus ribavirin (RBV) for 48 weeks for genotypes 1 and 4, and for 24 weeks for other genotypes [4–6]. Several host factors, such as gender, age, serum HCV RNA level, progression of liver fibrosis [7] and interleukin (IL)-28B [8,9], as well as virological factors such as virus genotype, the number of mutations in the interferon sensitivity determining region (ISDR) [10] and substitutions of amino acids 70 and 91 in the core region [11], have been useful for predicting the response to PEG-IFN/RBV therapy.

IL-6 is a pleiotropic cytokine that plays a role in the acute phase response [12]. IL-6 is released from various cells, that is, leukocytes, fibroblasts, endothelial cells and macrophages, in response to following systematic or local infection, tissue injury and inflammation [13]. As for the liver, IL-6 is produced mainly by Kupffer cells [14] and induces the production of the acute phase proteins, C-reactive protein and haptoglobin [12]. Previous studies reported that serum IL-6

Will There Be an HCV Meeting in 2020? Summary of the 17th International Meeting on Hepatitis C Virus and Related Viruses

TAKAJI WAKITA,* TETSURO SUZUKI,‡ MATTHEW J. EVANS,§ KUNITADA SHIMOTOHNO,‖ KAZUAKI CHAYAMA,¶ YOSHIHARU MATSUURA,# MAKOTO HIJIKATA,** KOHJI MORIISHI,‡‡ TSUKASA SEYA,§§ NOBUYUKI ENOMOTO,¶¶ KAZUHIKO KOIKE,## NOBUYUKI KATO,*** TATSUYA KANTO,# and HAK HOTTA‡‡‡

*National Institute of Infectious Diseases, Japan; ‡Hamamatsu University School of Medicine, Japan; §Mount Sinai School of Medicine, New York; ‖Chiba Institute of Technology, Japan; ¶Hiroshima University, Japan; #Osaka University, Japan; **Kyoto University, Japan; ¶¶University of Yamanashi, Japan; §§Hokkaido University, Japan; ##The University of Tokyo, Japan; ***Okayama University, Japan; ‡‡‡Kobe University, Japan

Hepatitis C virus (HCV), which was discovered in 1989, is a major etiologic agent in human liver disease. Approximately 130 million people, or 2% of the population, worldwide are infected. The 17th International Meeting on Hepatitis C Virus and Related Viruses was held September 10–14, 2010, in Yokohama, Japan. The meeting was attended by almost 700 scientists from all over the world who are interested in the fundamental aspects of the molecular virology, immunology, pathogenesis, prevention, and treatment of HCV infection. Two special opening lectures given by Masaaki Komatsu and Takashi Gojobori focused attention on the related research fields of autophagy and genome biology, respectively. In the subsequent sessions, the latest research, original studies, and controversies were presented in 9 keynote lectures, 82 oral presentations, and 329 poster presentations.

Viral Entry

The opening scientific session of this meeting focused on the viral host cell entry processes. Thomas Baumert presented the keynote lecture, which included an overview of the HCV cell entry process and recent advances at his laboratory. These included the finding that HCV variants that reinfect the liver after transplantation demonstrate more efficient cell entry and are less susceptible to neutralization by host antibodies. He also described the isolation of monoclonal antibodies against claudin-1 that do not inhibit either extracellular or direct cell-to-cell HCV transfer.

Alexander Ploss described the establishment of a mouse model for studying HCV cell entry. They utilized an HCV cell culture virus (HCVcc) expressing recombinase and transgenic mice bearing a recombinase-activatable fluorescent protein. Bioluminescent imaging indicated that only mice transduced with CD81 and occludin supported HCVcc entry. The presence of an intact immune system in these animals makes it particularly important for the testing of HCV vaccine candidates. Danyelle N. Martin described a role for transferrin receptor 1 (TfR1) in mediating HCV cell entry. The inhibition of HCV entry with TfR1 antibodies and silencing, suggest this factor should be added to the growing list of cellular proteins required for HCV cell entry. Joachim Lupberger presented results from a study showing an essential role for the epidermal growth factor receptor (EGFR) in HCV cell entry. He found that EGFR is required for both mediating the interactions between two other entry factors, CD81 and CLDN1, and catalyzing the fusion activity of viral glycoproteins.

Translation/Replication

Volker Lohmann began the session by describing what is known of the functions of viral nonstructural proteins and their associated host cellular factors in viral translation and replication. He included an overview of viral isolates and model systems currently used, and presented data addressing the mechanisms for efficient replication of the JFH-1 isolate.

Several reports have focused on the molecular basis of the architecture and composition of membrane-associated sites for HCV replication, which often induce membrane alterations, such as the so-called membranous web. Brenno Wolk demonstrated that NS4B is sufficient to direct all nonstructural proteins into the viral replication complex compartment, and that intragenotype-specific interactions are required for NS4B-dependent recruitment of NS5A. Ines Romero-Brey showed that the membranous web predominantly contains double-membrane vesicles with various diameters. These vesicle structures were connected to the endoplasmic reticulum (ER) through funnel-like structures.

Several DDX DEAD-box RNA helicases were identified as host factors associated with HCV replication. Yasuo Ariumi presented the cross-talk of HCV with DDX proteins and the role of distinct DDX proteins in viral replication. Tetsuro Shimakami and Selena M. Sagan reported the importance of miR-122 to not only enhance IRES-mediated translation, but stabilize positive-strand HCV RNA by binding to its 5′ extremity. Enzymatic activity of host phosphatidyl-inositol-4 kinase III alpha was shown to be critically involved in HCV replication and the activity is regulated by HCV NS5A (Simon Reiss). Nam-Joon Cho reconstituted a functionally active full-

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Antiviral activity of novel 2′-fluoro-6′-methylene-carbocyclic adenosine against wild-type and drug-resistant hepatitis B virus mutants

Jianing Wang [a], Uma S. Singh [a], Ravindra K. Rawal [a], Massaya Sugiyama [b], Jakyung Yoo [a], Ashok K. Jha [a], Melissa Scroggin [a], Zhuhui Huang [c], Michael G. Murray [c], Rajgopal Govindarajan [a], Yasuhito Tanaka [b], Brent Korba [d], Chung K. Chu [a,*]

[a] The University of Georgia, College of Pharmacy, Athens, GA 30602, USA
[b] Nagoya City University, Graduate School of Medical Sciences, Nagoya 467-8601, Japan
[c] Southern Research Institute, Frederick, MD 21701, USA
[d] Georgetown University Medical Center, Washington, DC 20057, USA

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ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (**9**) was synthesized and evaluated its anti-HBV activity. The titled compound demonstrated significant antiviral activity against wild-type as well as lamivudine, adefovir and double lamivudine/entecavir resistant mutants. Molecular modeling study indicate that the 2′-fluoro moiety by a hydrogen bond, as well as the van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug resistant mutants.

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Chronic hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality worldwide. Chronic infection with HBV occurs in approximately 350 million of the world population, including 1.7 million in the USA.[1] HBV infection can persist for the life of the host, often leading to severe consequences such as liver failure, cirrhosis and eventually hepatocellular carcinoma, resulting in annually 0.5–1.2 million deaths worldwide.[2] HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique because it includes a reverse transcription step. The HBV DNA polymerase/reverse transcriptase is an essential and multifunctional enzyme, which operates as a DNA polymerase/reverse transcriptase, an RNAse H, through coordinating the assembly of viral nucleocapsids, as well as catalyzing the generation of DNA primers.[3] Nucleoside analogues can suppress HBV replication by inhibiting the viral polymerase/reverse transcriptase. The pivotal role of nucleoside/nucleotide analogues such as lamivudine, adefovir, telbivudine, entecavir, clevudine, and tenofovir has been demonstrated by their therapeutic efficacy in clinical practice. However, long-term therapy with these drugs is often associated with viral resistance, which significantly compromises the clinical application of these agents. For example, the extensive use of lamivudine resulted in the emergence of mutants that are resistant to the anti-HBV activity; 24% after a 1-year therapy, increasing to over 70% after 4 years of therapy. Adefovir has been used for the patients, who develop lamivudine-resistant mutants, however, a significant number of patients (29% after 5 years of use) also develop the adefovir resistant mutant (N236T).

Entecavir is a carbocyclic 2′-deoxyguanosine analog that demonstrates potent anti-HBV activity[4] and is recommended for patients with the wild-type strain as well as for those patients harboring lamivudine-resistant strains.[5] However, a recent study by Tanaka and his co-workers suggest that the viral breakthrough was observed in the lamivudine-refractory group in 4.9% of patients at baseline and increase to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[6]

In view of the fact that currently adefovir and entecavir are the most prescribed drugs for the treatment of chronic HBV infection, it is critical to discover the agents that do not confer cross-resistance with the adefovir and lamivudine/entecavir-mutants for the future treatment of drug resistant patients. In this report we try to demonstrate that our newly discovered compound **9** may potentially play a significant role for that purpose.

Carbocyclic nucleosides are an interesting class of compounds in which the methylene group replaces the oxygen atom of a furanose ring. As a consequence, the glycosidic bond is resistant to nucleoside phosphorylase as well as nucleoside hydrolase, which makes the carbocyclic nucleosides more stable towards metabolic

* Corresponding author. Address: Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602, USA. Tel.: +1 706 542 5379; fax: +1 706 542 5381.
E-mail addresses: DCHU@rx.uga.edu, DCHU@mail.rx.uga.edu (C.K. Chu).

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Inhibitory Effect of a Triterpenoid Compound, with or without Alpha Interferon, on Hepatitis C Virus Infection▽†

Takako Watanabe,[1]‡ Naoya Sakamoto,[1,2]‡* Mina Nakagawa,[1,2] Sei Kakinuma,[1,2] Yasuhiro Itsui,[3] Yuki Nishimura-Sakurai,[1] Mayumi Ueyama,[1] Yusuke Funaoka,[1] Akiko Kitazume,[1] Sayuri Nitta,[1] Kei Kiyohashi,[1] Miyako Murakawa,[1] Seishin Azuma,[1] Kiichiro Tsuchiya,[1] Shinya Ooka,[1] and Mamoru Watanabe[1]

Department of Gastroenterology and Hepatology[1] and Department for Hepatitis Control,[2] Tokyo Medical and Dental University, Tokyo, Japan, and Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan[3]

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A lack of patient response to alpha interferon (α-IFN) plus ribavirin (RBV) treatment is a major problem in eliminating hepatitis C virus (HCV). We screened chemical libraries for compounds that enhanced cellular responses to α-IFN and identified a triterpenoid, toosendanin (TSN). Here, we studied the effects and mechanisms of action of TSN on HCV replication and its effect on α-IFN signaling. We treated HCV genotype 1b replicon-expressing cells and HCV-J6/JFH-infected cells with TSN, with or without α-IFN, and the level of HCV replication was quantified. To study the effects of TSN on α-IFN signaling, we detected components of the interferon-stimulated gene factor 3 (ISGF3), phosphorylated signal transducer and activator of transcription 1 (STAT1), and STAT2 by Western blotting analysis; expression levels of mRNA of interferon regulatory factor 9 using real-time reverse transcription-PCR (RT-PCR); and interferon-stimulated response element reporter activity and measured the expression levels of interferon-inducible genes for 2′,5′-oligoadenylate synthetase, MxA, protein kinase R, and p56 using real-time RT-PCR. TSN alone specifically inhibited expression of the HCV replicon (50% effective concentration = 20.6 nM, 50% cytotoxic concentration > 3 μM, selectivity index > 146). Pretreatment with TSN prior to α-IFN treatment was more effective in suppressing HCV replication than treatment with either drug alone. Although TSN alone did not activate the α-IFN pathway, it significantly enhanced the α-IFN-induced increase of phosphorylated STATs, interferon-stimulated response element activation, and interferon-stimulated gene expression. TSN significantly increased baseline expression of interferon regulatory factor 9, a component of interferon-stimulated gene factor 3. Antiviral effects of treatment with α-IFN can be enhanced by pretreatment with TSN. Its mechanisms of action could potentially be important to identify novel molecular targets to treat HCV infection.

Hepatitis C virus (HCV) is one of the most important pathogens causing acute and chronic hepatitis, liver cirrhosis, and hepatocellular malignancies (29). Alpha interferon (α-IFN) combined with ribavirin (RBV) is the standard treatment for HCV infection (6, 10). However, virus elimination rates are about 50% among treated patients, and therapy is often accompanied by substantial side effects (6, 44). It was recently reported that genetic polymorphisms of the *IL28B* gene, which codes for lambda IFN, are critical for predicting responses to α-IFN plus RBV therapy (8, 35, 38). Patients with minor variants of *IL28B*, who comprise ~50% of Caucasian, 25% of Asian, and ~70% of African populations, showed poor responses to α-IFN treatment. Although new specific anti-HCV drugs are under development, many of them require combined use with α-IFN and RBV (26). Taken together, current difficulties in eliminating HCV are mostly attributable to the limited treatment options and to the limited activity of α-IFN against the virus. For this reason, the development of safe and effective agents that enhance antiviral actions against HCV has been a strong motivation in academia and industry.

To search for a new agent which enhances the effect of α-IFN, we used interferon-stimulated response element (ISRE) reporter screening. We screened a chemical library (60,500 compounds) for compounds that enhance ISRE activity when they are used in combination with α-IFN, using ISRE reporter screening, and identified several compounds that increased the ISRE reporter activities when they are used in combination with α-IFN and that did not show cytotoxicity. Among the hit compounds, toosendanin (TSN; $C_{30}H_{38}O_{11}$; molecular weight = 574) (Fig. 1), which is a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb et Zucc, was the strongest in enhancing α-IFN-induced ISRE reporter activation and the expression of interferon-stimulated genes (ISGs). TSN has been used as an anthelmintic vermifuge against ascaris (31). Although TSN has some other biological effects against toxin-producing anaerobic bacteria and against carcinoma cells (32, 45), antiviral activity has not been reported.

In this study, we showed, using an HCV replicon system, that TSN, with or without α-IFN, inhibits HCV replication in a cultured human hepatoma Huh7 cell line and that the combination of TSN and α-IFN shows synergistic effects on viral replication. We have investigated the mechanisms of action of

* Corresponding author. Mailing address: Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81 3-5803-5877. Fax: 81 3-5803-0268. E-mail: nsakamoto.gast@tmd.ac.jp.

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‡ T.W. and N.S. contributed equally to this work.

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