BASIC AND TRANSLATIONAL—LIVER

Thromboxane A_2 Synthase Inhibitors Prevent Production of Infectious Hepatitis C Virus in Mice With Humanized Livers

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See Covering the Cover synopsis on page 495.

BACKGROUND & AIMS: A 3-dimensional (3D) culture system for immortalized human hepatocytes (HuS-E/2 cells) recently was shown to support the lifecycle of blood-borne hepatitis C virus (HCV). We used this system to identify proteins that are active during the HCV lifecycle under 3D culture conditions. **METHODS:** We compared gene expression profiles of HuS-E/2 cells cultured under 2-dimensional and 3D conditions. We identified signaling pathways that were activated differentially in the cells, and analyzed their functions in the HCV lifecycle using a recombinant HCV-producing cell-culture system, with small interfering RNAs and chemical reagents. We investigated the effects of anti-HCV reagents that altered these signaling pathways in mice with humanized livers (carrying human hepatocytes). **RESULTS:** Microarray analysis showed that cells cultured under 2-dimensional vs 3D conditions expressed different levels of messenger RNAs encoding prostaglandin synthases. Small interfering RNA–mediated knockdown of thromboxane A2 synthase (TXAS) and incubation of hepatocytes with a TXAS inhibitor showed that this enzyme is required for production of infectious HCV, but does not affect replication of the HCV genome or particle release. The TXAS inhibitor and a prostaglandin I2 receptor agonist, which has effects that are opposite those of thromboxane A_2, reduced serum levels of HCV and inhibited the infection of human hepatocytes by blood-borne HCV in mice. **CONCLUSIONS: An inhibitor of the prostaglandin synthase TXAS inhibits production of infectious HCV particles in cultured hepatocytes and HCV infection of hepatocytes in mice with humanized livers. It therefore might be therapeutic for HCV infection.**

Keywords: Infectious Virus Particle; Lipid Mediator; Antiviral Drug.

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV),[1] with the majority suffering from chronic hepatitis, cirrhosis, and/or hepatocellular carcinoma.[2] HCV currently is treated using a combination of polyethylene glycol–conjugated interferon and ribavirin, although no more than 60% of individuals adequately respond.[3] Recently, inhibitors of HCV nonstructural proteins have been developed as direct-acting antiviral agents to treat HCV effectively.[4–6] However, HCV often acquires resistance against treatment with direct-acting antiviral agents in cases of monotherapy.[7] Current efforts therefore are focused on better understanding the lifecycle of HCV to find the cellular target of novel anti-HCV drugs to use the options for multi-drug therapy.

A cell-culture system that allows the production of recombinant infectious HCV (HCVcc) recently was developed using a cloned HCV genome and the hepatocellular carcinoma–derived Huh-7 cell line.[8–10] Experiments using the culture system have provided novel insights on the HCV lifecycle such as finding the production of infectious HCV particles near lipid droplets (LDs) and endoplasmic reticulum–derived LD-associated membranes.[11] Huh-7 cells, however, only allow the proliferation of recombinant HCV, and not blood-borne HCV (bbHCV).

To study the lifecycle of bbHCV, we cloned immortalized human hepatocyte HuS-E/2 cells, which permitted some degree of bbHCV infection.[12] Integrating hollow fibers into the 3-dimensional (3D) culture system resulted in efficient continuous proliferation of infected HCV production from the cells.[13] By using the improved system, we previously compared the gene expression profiles of HuS-E/2 cells under the 2-dimensional (2D) and 3D culture conditions using microarray analysis. This allowed us to identify signaling pathways that contribute to the proliferation of HCV, for example, peroxisome proliferator–activated

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; AAC, arachidonic acid cascade; bbHCV, blood-borne hepatitis C virus; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; HCV, hepatitis C virus; HCVcc, hepatitis C virus from cell culture; IP, prostaglandin I_2 receptor; LD, lipid droplet; mRNA, messenger RNA; PG, prostaglandin; PGIS, prostaglandin I_2 synthase; RT-PCR, reverse-transcription polymerase chain reaction; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2; TXAS, thromboxane A_2 synthase; TXB_2, thromboxane B_2.

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receptor α signaling, which enhances HCV replication.[14] This result was confirmed by other groups,[15,16] corroborating that our strategy can uncover cellular events that support the proliferation of HCV. We therefore hypothesized that leveraging the in vitro systems described earlier may help elucidate the molecular mechanisms underlying the HCV lifecycle.

Prostanoids are metabolites of the arachidonic acid cascade (AAC) that possess various physiologic activities.[17] These metabolites include prostaglandin (PG) E_2, D_2, I_2, and F_2, and thromboxane A_2 (TXA_2).[17] Although several studies have shown that PG signaling contributes to liver regeneration,[18,19] the physiologic functions of these lipid mediators in human hepatocytes still are unknown. Interestingly, one report showed that PGE_2 might support HCV genome replication in cells bearing self-replicating HCV subgenomic replicon RNA.[20] Whether prostanoids are involved in the HCV lifecycle, however, has not been precisely investigated.

In this study, we provide evidence that TXA_2 synthase (TXAS) is involved in the formation of infectious HCV, by cell culture system, and that a TXAS inhibitor and PGI_2 receptor (IP) agonist that has opposite physiological effects to TXA_2 can be used as a novel anti-HCV drug by using chimeric mice bearing transplanted human hepatocytes.[21] This report shows the contribution of the AAC to HCV infectivity and the potency of a prostanoid as an antiviral agent.

Materials and Methods

Cell Culture

The human hepatocellular carcinoma–derived Huh-7 and Huh-7.5 cell lines were cultured as described previously.[22] HuS-E/2 cells are immortalized human hepatocytes transduced with *E6* and *E7* genes of human papilloma virus 18 and human telomerase reverse-transcription gene as described previously.[12] The 2D and 3D culture conditions for HuS-E/2 cells were as described previously.[12]

Reagents and Antibodies

FR122047, PGH_2, ONO1301, daltroban, and dibutyryl cyclic adenosine monophosphate (cAMP) sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Cyclooxygenase (COX)-2 inhibitor 1 and Ozagrel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U-46619 was purchased from Cayman Chemical (Ann Arbor, MI). Beraprost was a generous gift from Toray, Co (Tokyo, Japan). FR122047, PGH_2, ONO1301, Daltroban, COX-2 inhibitor 1, Ozagrel, Beraprost, and calcium ionophore were dissolved in dimethyl sulfoxide. U-46619 and thromboxane B_2 (TXB_2) were dissolved in methyl acetate. Dibutyryl cAMP was dissolved in water. The effect of each reagent on cell viability was analyzed using a Cell Proliferation Kit 2 (Roche, Basel, Switzerland) based on the manufacturer's instructions. An antibody specific for core protein (antibody 32-1) was a gift from Dr Michinori Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Rabbit polyclonal anti-NS5A protein CL1 antibody and anti-HCV protein antibody in human serum were described previously.[11]

Microarray Analysis

Total RNA purified from HuS-E/2 cells cultured under 2D or 3D conditions in the absence of HCV infection was analyzed with a 3D-Gene Human Chip 25k (Toray, Co) to compare gene expression profiles as described previously.[14] The accession number of the results is listed as "E-MTAB-1491" in ArrayExpress.

Production of HCVcc and Sample Preparation

HCVcc was produced from the Huh-7 or Huh-7.5 cells transfected with in vitro synthesized Jikei Fulminant Hepatitis (JFH) 1^{E2FL} or J6/JFH1 RNA as described previously.[11] The transfected cells and culture medium were harvested at 4 days post-transfection. For $JFH1^{E2FL}$ RNA-transfected Huh-7 cells treated with TXAS-specific small interfering RNA (siRNA), cells and culture medium were harvested 3 days after transfection. Culture medium including HCVcc was concentrated and used for infection experiments as described previously.[11] Concentrated culture medium from JFH1 RNA-transfected Huh-7 cells was fractionated as described previously.[11] The infectivity titer in each fraction was analyzed by focus-formation assay, which was determined by the average number of HCV-positive foci.

Reverse-Transcription Polymerase Chain Reaction and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from the cells and medium using Sepasol I Super and Sepasol II (Nacalai Tesque, Kyoto, Japan), respectively, according to the manufacturer's instructions. By using 200 ng of total RNA as a template, we performed reverse-transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) with a 1-step RNA PCR kit and a 1-step SYBR Primescript RT-PCR kit 2 (Takara, Shiga, Japan), respectively, according to the manufacturer's instructions. Information on both experiments is shown in Supplementary Tables 1 and 2.

Infection of HCVcc

Infection experiments of HCVcc and detection of infected Huh-7.5 cells by indirect immunofluorescence analysis were performed mainly as described previously.[11] The number of infection-positive cells detected in 4×10^4 target cells 1 day after infection with HCVcc including 10^7 copies of RNase-resistant HCV genome was defined as the specific infectivity in the infection experiments in our protocol.

Indirect Immunofluorescence Analysis

HCV proteins were examined in cells using a Leica SP2 confocal microscope (Leica, Heidelberg, Germany), and infected cells were counted using a BioZero fluorescence microscope (Keyence, Tokyo, Japan).

Preparation of Intracellular HCV Particles

Intracellular HCV particles were prepared as described previously.[23]

Pharmacologic Test in Chimeric Mice Bearing Transplanted Human Hepatocytes

All mouse studies were conducted at Hiroshima University (Hiroshima, Japan) in accordance with the guidelines of the local committee for animal experiments. Chimeric mice