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Short communication

Inhibition of porcine endogenous retrovirus (PERV) replication by HIV-1 gene expression inhibitors

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ABSTRACT

Porcine endogenous retrovirus (PERV) is persistently integrated into the host genomic DNA as a provirus and released from a variety of porcine cells. PERV infects a certain range of human cells, which is a major concern in xenotransplantation. Therefore, the use of viral gene expression inhibitors could be envisaged, if they reduce PERV production from porcine organs and minimize viral transmission to human recipients. In the present study, four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in porcine cells constitutively producing the virus. Among the compounds, the fluoroquinolone derivative K-37 and the bacterial product EM2487 displayed potent and selective inhibition of PERV replication in the cells mediated by the suppression of viral mRNA synthesis. Thus, retroviral gene expression inhibitors may be able to reduce the risk of PERV transmission.

Xenotransplantation, the grafting of cells, tissues, or organs into different species, is a possible solution to overcome the extreme shortage of human allografts for transplantation (Cooper and Keogh, 2001). Among the animals, non-human primates and pigs are considered to be suitable donors for xenotransplantation. The use of non-human primates as organ donors is associated with a high risk of transmitting various infectious pathogens to humans (Allan, 2003). Apart from immunological rejection, pigs may be more suitable donors than non-human primates because of the resemblance of their organ sizes and a lower risk of transmitting various infectious pathogens. However, porcine endogenous retrovirus (PERV) is still a major obstacle to successful xenotransplantation with sufficient safety. PERV is a type C retrovirus persistently integrated into the host genomic DNA as a provirus. Multiple copies of PERV proviral DNA exist in all of the breeds examined to date (Louz et al., 2008). PERV is classified into three subtypes, such as PERV-A, -B, and -C, based on the divergence of its envelope genes.

It has been demonstrated that PERV particles are released from a variety of porcine cells and infect a certain range of human cells (Martin et al., 1998; Patience et al., 1997; Wilson et al., 1998). There are a number of patients who received porcine tissues, such as pancreatic islet cells, skin, liver, and kidney; nevertheless PERV infection has not been observed in these individuals (Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998). However, long-lived microchimerism was found in some patients treated by extracorporeal splenic perfusion, which might increase a potential risk of PERV infection through the activation of viral replication (Paradis et al., 1999). An immunosuppressive treatment upon organ transplantation may also increase a risk of PERV transmission. The use of antiretrovirals would be the first option to minimize the possibility of PERV transmission to recipients, if they could have an inhibitory effect on PERV replication without serious side effects. Among the antiretrovirals, zidovudine (AZT) and didanosine (ddI) proved to be active against PERV replication in cell cultures (Powell et al., 2000; Qari et al., 2001). We have previously demonstrated that the acyclic nucleoside phosphonate tenofovir (PMPA), an HIV-1 reverse transcriptase (RT) inhibitor, selectively inhibits PERV replication in human cells (Shi et al., 2007). However, such RT inhibitors cannot suppress the production of PERV from the porcine cells in which its proviral DNA is integrated. Therefore, it would be very useful if an inhibitor of PERV gene expression could be identified. In the present study, we have examined four inhibitors of HIV-1 gene expression for their antiviral activity against PERV replication in porcine cells persistently infected with the virus and found that the fluoroquinolone derivative K-37 (Baba et al., 1998) and the bacterial product EM2487 (Baba et al., 1999) are potent and selective inhibitors of PERV replication.

K-37 and the nuclear factor κB (NF-κB) inhibitor cepharanthine (Okamoto et al., 1998) were provided by Daiichi Pharmaceutical

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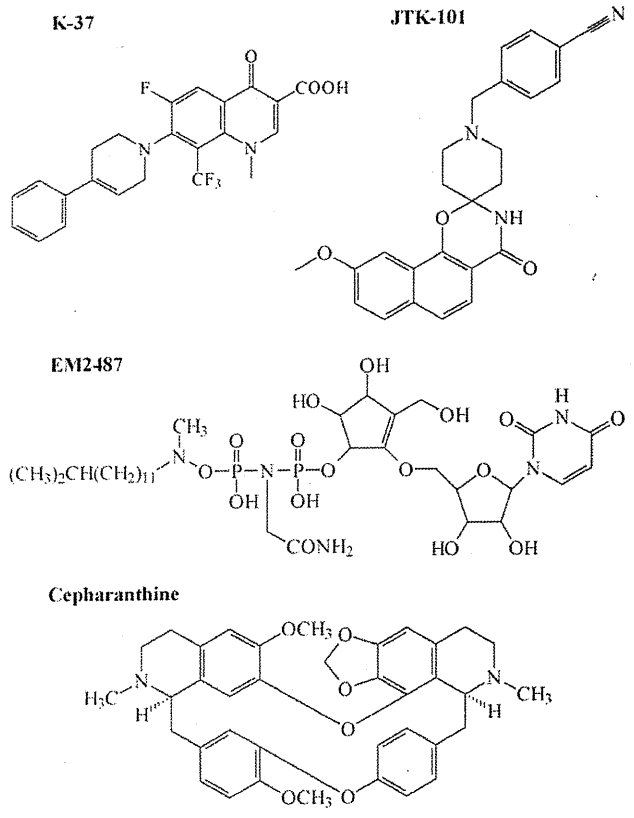

Fig. 1. Chemical structures of test compounds.

Co. (Tokyo, Japan) and Kaken Shoyaku (Mitaka, Japan), respectively. JTK-101 (Wang et al., 2007) was synthesized by Japan Tobacco Co. (Takatsuki, Japan). EM2487 was provided by Esai Co. (Tsukuba, Japan). These compounds (Fig. 1) were chosen for this study, because their antiviral activity against HIV-1 replication in chronically infected cells had been demonstrated (Baba et al., 1998, 1999; Okamoto et al., 1998; Wang et al., 2007). All compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of DMSO and stored −20 °C until use. The porcine embryonic kidney cell line PK15, which produces PERV particles, was obtained from the American Type Culture Collection. The cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and antibiotics.

The activity of the compounds against persistent PERV infection was based on the inhibition of PERV particle production from PK15 cells. PK15 cells were seeded in a 24-well plate (2.5×10^4 cells/well). After incubation for 16 h at 37 °C, the culture supernatants were removed and the cell monolayer was washed by phosphate-buffered saline (PBS), and then 2 ml of fresh medium containing various concentrations of the test compounds was added to each well. After a 48-h incubation period, the culture supernatants were collected and filtered (0.45 μm pore size). Then the filtrates were mixed with 22% (w/v) polyethylene glycol 6000 solution. After incubation for 5 h at 4 °C with continuous stirring, the mixture was centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets contained PERV particles released from PK15 cells. The inhibition of PERV particle production was determined by the decrease of PERV reverse transcriptase activity using a commercial RT assay kit (Roche, Mannheim, Germany). The pellets obtained above were resuspended in lysis buffer supplied by the assay kit and subjected to reverse transcription reaction for 2 h, according to the Manufacturer's instructions, except that $MgCl_2$ in the reaction mixture was replaced by $MnCl_2$ (Phan-Thanh et al., 1992). All experiments were carried out in duplicate.

The antiviral activity of test compounds was also determined by the inhibition of PERV mRNA expression in PK15 cells. PK15 cells were seeded and cultured in the medium containing test compounds in the same manner as described for the antiviral assay. After a 48-h incubation, the culture supernatants were removed, and the cells were extensively washed with PBS, trypsinized, and washed again with PBS. Total RNA was extracted from the cells with RNeasy Mini Kit (Qiagen) and subjected to real-time RT-PCR analysis. The PERV mRNA level was determined using the sense primer (5′-AGCTCCGGGAGGCCTACTC-3′), the antisense primer (5′-ACAGCCGTTGGTGTGGTCA-3′), and the Taqman® probe (5′-FAM-CCACCGTGCAGGAAACCTCGAGACT-TAMRA-3′). The primer pair amplifies a region of the *pol* gene of PERV (Paradis et al., 1999). The nucleotide sequences used for the construction of the primers and probe were based on the reports by B. Bartosch, R.A. Weiss and Y. Takeuchi (GeneBank accession numbers: AY099323 and AY099324). The final concentrations of the primer pairs and probe were 200 and 100 nM, respectively. The Taqman® PCR reagent kit and Taqman® Multiscribe™ reverse transcription reagent kit (Applied Biosystems, Roche, Branchburg, NJ) were used according to the Manufacturer's instructions. Each sample was run in triplicate. Nonspecific inhibition of host cellular mRNA synthesis by the test compounds was determined with the Taqman 18S rRNA reagent kit (Applied Biosystems).

Cytotoxicity of the test compounds was determined by a tetrazolium dye method (Tetracolor One®, Seikagaku Corporation, Tokyo, Japan) (Yamamoto et al., 2001). PK15 cells were seeded and cultured in the medium containing test compounds in the same manner, as described in the antiviral assay. After a 48-h incubation, 1.5 ml of the culture supernatants were removed and 25 μl of the dye was added to each well. After a 4-h incubation at 37 °C, the specific (450 nm) and reference (630 nm) absorbances were monitored for each well by a microplate reader.

When four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in PK15 cells, K-37 and EM2487 displayed dose-dependent reduction of PERV RT activity in culture supernatants (Fig. 2A and C). K-37 and EM2487 did not show a direct inhibitory effect on PERV RT activity (data not shown). These compounds did not display apparent cytotoxicity to PK15 cells at concentrations up to 1 and 10 μM, respectively, indicating that K-37 and EM2487 are selective inhibitors of PERV replication in porcine cells. In contrast, JTK-101 and cepharanthine did not show any activity against PERV replication at the highest concentration tested (1 μM) (Fig. 2B and D). Since PERV proviral DNA is integrated in the genome of the host cells, the compounds were also examined for their inhibitory effect on viral mRNA synthesis in PK15 cells. As shown in Fig. 3, dose-dependent suppression of PERV mRNA synthesis was observed for K-37 and EM2487 but not for JTK-101 or cepharanthine. These results are in accordance with those obtained in the RT assay (Fig. 2). The 50% effective concentration (EC_{50}) of K-37 for PERV replication and its 50% inhibitory concentration (IC_{50}) for viral mRNA synthesis were 0.35 ± 0.04 and 0.34 ± 0.05 μM, respectively (Table 1). On the other hand, its 50% cytotoxic concentration (CC_{50}) was 4.63 ± 1.62 μM, suggesting that K-37 is a selective inhibitor of PERV gene expression. Similarly, the EC_{50}, IC_{50}, and CC_{50} of EM2487 were 5.44 ± 1.40, 4.36 ± 0.30, and >10 μM, respectively.

K-37 is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells at submicromolar concentrations (Baba et al., 1998). K-37 could inhibit Tat-dependent transactivation, yet it was not an inhibitor of Tat itself or its cofactor CDK9/cyclin T1. Since PERV does not generate Tat protein, it is apparent that the anti-PERV activity of K-37 is not due to the inhi-

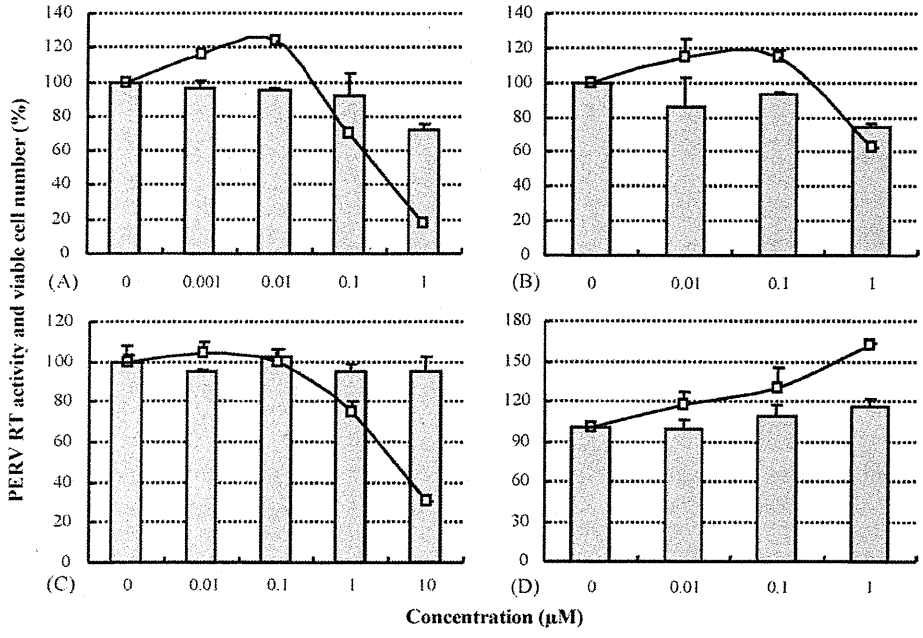

Fig. 2. Inhibitory effects of the test compounds on PERV replication in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation period, the culture supernatants were collected and mixed with 22% (w/v) polyethylene glycol 6000 solution for 5 h. PERV particles were harvested by centrifugation of the mixture. The viral pellets were resuspended in lysis buffer and subjected to RT assay (lines). The viable cell number was determined by a tetrazolium dye cell proliferation assay (bars). Both the RT activity and cell proliferation assays were performed in duplicate. The data represent means plus ranges. Representative results for two independent experiments are shown.

bition of Tat functions. Furthermore, K-37 was reported to inhibit the gene expression of human T-lymphotropic virus type 1 (HTLV-1) in persistently infected cells (Wang et al., 2002a). Although the target molecule of K-37 still remains to be determined, the present observations for PERV suggest that K-37 may interact with a cellular factor or factors that play an important role in retroviral gene expression. It is assumed that K-37 inhibits an early stage of transcriptional elongation of viral RNA (Okamoto et al., unpublished observations). EM2487 is a substance produced from a *Streptomyces* species and a potent and selective inhibitor of HIV-1 replication in acutely and chronically infected cells (Baba et al., 1999). Like K-37, EM2487 could inhibit HTLV-1 gene expression without affecting host cellular functions (Wang et al., 2002b). The chemical structures of K-37 and EM2487 are totally different from each other

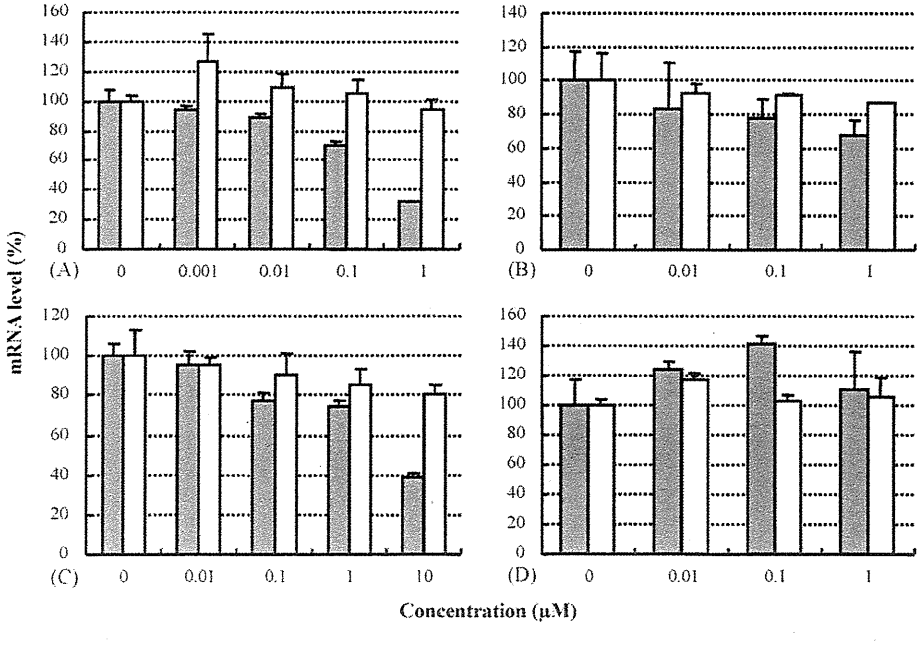

Fig. 3. Inhibitory effect of the test compounds on PERV mRNA synthesis in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation, the cells were collected, and total RNA was extracted. Quantitative real-time RT-PCR was performed to determine the amount of PERV mRNA in PK15 cells using a primer pair and probe specific to the PERV *pol* gene (gray columns). The inhibitory effect of the test compounds on host cellular mRNA synthesis was determined by quantitative RT-PCR for 18S mRNA (white columns). All experiments were performed in triplicate. The data represent means plus standard deviations. Representative results for two independent experiments are shown.

Table 1
Inhibitory effect of test compounds on PERV antigen production and mRNA synthesis in PK15 cells[a].

Compounds	PERV[b]			HIV-1[c]		HTLV-1[d]	
	EC_{50} (μM)	IC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
K-37	0.35 ± 0.04	0.34 ± 0.05	4.63 ± 1.62	0.033 ± 0.012	2.1 ± 0.3	0.44 ± 0.13	5.7 ± 1.0
EM2487	5.44 ± 1.40	4.36 ± 0.30	>10	0.075 ± 0.032	12.5 ± 4.1	3.6 ± 0.6	30.6 ± 3.5
JTK-101	>1	>1	2.18 ± 0.63	0.0014 ± 0.0005	3.8 ± 0.2	N.D.[e]	N.D.
Cepharanthine	>1	>1	4.39 ± 1.99	0.028 ± 0.016	1.3 ± 0.3	>3.0	3.0 ± 0.4

[a] Each experiment was carried out in duplicate or triplicate, and all data represent means ± ranges for two independent experiments.
[b] EC_{50}: 50% effective concentration based on the inhibition of PERV antigen production (RT) in culture supernatants of PK15 cells. IC_{50}: 50% inhibitory concentration based on the inhibition of PERV mRNA synthesis. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation.
[c] EC_{50}: 50% effective concentration based on the inhibition of HIV-1 antigen production (p24) in chronically infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2007) for K-37 and JTK-101, Baba et al. (1999) for EM2487, and Baba et al. (2001) for cepharanthine.
[d] EC_{50}: 50% effective concentration based on the inhibition of HTLV-1 antigen production (p19) in infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2002a) for K-37 and cepharanthine and Wang et al. (2002b) for EM2487.
[e] Not determined.

(Fig. 1), nevertheless they appear to share some common properties in antiretroviral activity and mechanism of action.

JTK-101 is a novel naphthalene derivative that inhibits HIV-1 replication in cell cultures (Wang et al., 2007). This compound was found to be highly active against HIV-1 in chronically infected cells but much less active in acutely infected cells. Studies of its mechanism of action suggested that JTK-101 exerted its anti-HIV-1 activity through the inhibition of CDK9/cyclin T1. Cepharantine is a plant alkaloid that has been shown to inhibit HIV-1 replication in a certain chronically infected cell line at low concentrations through the inhibition of NF-κB (Okamoto et al., 1998). Cepharanthine could also suppress stimulation-induced production of proinflammatory cytokines in human macrophages (Okamoto et al., 2001). This compound did not inhibit PERV replication in PK15 cells or even slightly enhanced it at the highest concentration tested (Fig. 2D).

The viral gene expression inhibitors K-37 and EM2487 may be able to keep PERV silent in porcine organs thereby reducing the risk of PERV transmission to recipients, which is never attainable with RT inhibitors. On the other hand, RT inhibitors are capable of inhibiting De Novo infection of recipients with PERV derived from porcine organs. Thus, an ideal strategy to prevent PERV transmission to organ recipients may be the combined treatment with an RT inhibitor for recipients and a gene expression inhibitor for donor organs. Unfortunately, the current gene expression inhibitors, such as K-37 or EM2487, may be toxic to human recipients at concentrations that completely suppress PERV production from donor cells or organs. Therefore, the optimization of their chemical structures would be required for the inhibition of PERV replication in vivo without generating serious side effects. Although the risk of PERV transmission upon xenotransplantation is supposed to be lower than initially thought, optimized retroviral gene expression inhibitors may be worth further pursuing for their potential efficacy in the clinical setting.

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Proanthocyanidin from Blueberry Leaves Suppresses Expression of Subgenomic Hepatitis C Virus RNA*[S]

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Hepatitis C virus (HCV) infection is a major cause of chronic liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. While searching for new natural anti-HCV agents in agricultural products, we found a potent inhibitor of HCV RNA expression in extracts of blueberry leaves when examined in an HCV subgenomic replicon cell culture system. This activity was observed in a methanol extract fraction of blueberry leaves and was purified by repeated fractionations in reversed-phase high-performance liquid chromatography. The final purified fraction showed a 63-fold increase in specific activity compared with the initial methanol extracts and was composed only of carbon, hydrogen, and oxygen. Liquid chromatography/mass-ion trap-time of flight analysis and butanol-HCl hydrolysis analysis of the purified fraction revealed that the blueberry leaf-derived inhibitor was proanthocyanidin. Furthermore, structural analysis using acid thiolysis indicated that the mean degree of polymerization of the purified proanthocyanidin was 7.7, consisting predominantly of epicatechin. Proanthocyanidin with a polymerization degree of 8 to 9 showed the greatest potency at inhibiting the expression of subgenomic HCV RNA. Purified proanthocyanidin showed dose-dependent inhibition of expression of the neomycin-resistant gene and the NS-3 protein gene in the HCV subgenome in replicon cells. While characterizing the mechanism by which proanthocyanidin inhibited HCV subgenome expression, we found that heterogeneous nuclear ribonucleoprotein A2/B1 showed affinity to blueberry leaf-derived proanthocyanidin and was indispensable for HCV subgenome expression in replicon cells. These data suggest that proanthocyanidin isolated from blueberry leaves may have potential usefulness as an anti-HCV compound by inhibiting viral replication.

Hepatitis C virus (HCV)[2] is often associated with the development of chronic liver diseases. Infection by HCV causes chronic hepatitis at high rates and finally results in liver cirrhosis and subsequent occurrence of hepatocellular carcinoma (1–3). The number of people worldwide who are infected by HCV is estimated to be over 200 million with 2 million infections in Japan (4). The South Kyushu area of Japan, including Miyazaki prefecture, has a high prevalence of this virus, and it is now recognized as a social problem. There is no vaccine effective for HCV at present. The elimination of HCV may be achieved by a combination of pegylated α-interferon and ribavirin, a broad spectrum antiviral drug (4–6). However, virological response to this combination therapy has been reported to be 80% for genotypes 2 and 3, but less than 50% for genotype 1 (7, 8). Moreover, α-interferon is associated with severe side-effects, including leucopenia, thrombocytopenia, depression, fatigue, and flu-like symptoms, and ribavirin is associated with side-effects such as hemolytic anemia (9). Therefore, establishment of a new modality of treatment without serious adverse effects is still required.

Considering the prolonged period (20–30 years) required for development of liver cirrhosis and hepatocellular carcinoma in individuals infected with HCV, we speculated that progression of the disease might be influenced by daily diet. Our research project focuses on the daily use of agricultural products that could cure or reduce the risk of disease progression by HCV. Thus, we screened local agricultural products (1700 samples from 283 species) for their suppressive activity against HCV subgenome expression using an HCV replicon cell system. We found a significant suppressive activity in extracts of blueberry leaves. Blueberries are classified in the genus *Vaccinium*, and the species are native only to North America. Blueberry leaves have high quinic acid and chlorogenic acid contents and also significant flavonol glycosides such as rutin. Thus, they are high in antioxidant activity. In our subsequent screening studies using various kinds of blueberry species, the most potent activity was observed in the leaf of rabbit-eye blueberry (*Vaccinium virgatum* Aiton), which is cultivated in southern areas of Japan.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: HCV, hepatitis C virus; hnRNP, heterogeneous nuclear ribonucleoprotein; HPLC, high-performance liquid chromatography; PDA, photodiode array; EPMA, electron probe micro-analysis; LC/MS-IT-TOF, liquid chromatography/mass spectrometry-ion trap-time of flight; APCI, atmospheric pressure chemical ionization; mDP, mean degree of polymerization; IC_{50}, concentration required for 50% inhibition; CC_{50}, concentration required for 50% cytotoxicity; eIF3, eukaryotic translation initiation factor 3; CHAPS, 3-[(3-cholamidopropryl)dimethylammonio]-1-propane sulfonate; IRES, internal ribosome entry site; DIGE, differential gel electrophoresis.

In this study, extracts of rabbit-eye blueberry leaves were used in an effort to purify and identify the compound responsible for inhibition of the expression of subgenomic HCV RNA. We identified oligomeric proanthocyanidin with mean degree of polymerization (mDP) around eight as an inhibitor of HCV subgenome expression. We also analyzed cellular proteins that have affinity to the oligomeric proanthocyanidin in HCV replicon cells and identified heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 as one of candidate proteins involved in the proanthocyanidin-mediated inhibition of HCV subgenome expression.

EXPERIMENTAL PROCEDURES

Extraction of Blueberry Leaves—A lyophilized powder made from leaves of rabbit-eye blueberry (*V. virgatum* Aiton) was provided by Unkai Shuzo Co., Ltd. (Miyazaki, Japan). One gram of the lyophilized powder was extracted with 100 ml of methanol at room temperature with shaking for 15 min, and the supernatant was passed through filter paper (filter paper No.2, Toyo, Tokyo, Japan). The methanol extract was then extracted with 100 ml of chloroform, followed by centrifugation (1750 × *g* for 10 min), and the resultant precipitate and supernatant were collected. The precipitate was dissolved in methanol, concentrated *in vacuo*, and lyophilized (CMW-ppt). The supernatant was mixed with 150 ml of distilled water and methanol to perform a liquid-liquid extraction, and the water layer was collected and mixed with 150 ml of chloroform to repeat the chloroform extraction. The water layer was concentrated and lyophilized (CMW-W). The chloroform layer was also concentrated and lyophilized (CMW-C). Most HCV subgenome-expression inhibitory activity was recovered in the CMW-W fraction.

Preparative Fractionation by HPLC—To separate the components in the CMW-W fraction processing inhibitory activity against HCV RNA expression, we performed HPLC (Prominence System, Shimadzu, Kyoto, Japan). Preliminary fractionation of CMW-W to confirm the elution pattern of HCV expression suppressive components was carried out on a reversed-phase column (Atlantis dC18, 4.6 mm × 150 mm, 3 μm, Waters, Milford, MA) at 40 °C with UV detection at 254 nm. A gradient consisting of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 15–25% B linear from 0 to 12.5 min, 25–100% B linear from 12.5 to 17.5 min followed by washing 100% B from 17.5 to 25 min. For purification, the first HPLC fractionation was performed on a reversed-phase column (Atlantis T3, 4.6 mm × 150 mm, 3 μm, Waters). A gradient consisting of eluant A and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 30% B from 0 to 7.5 min, 30–100% B linear gradient from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. The CMW-W fraction dissolved in 30 ml of methanol was injected, and the eluted fractions (2.1 to 18.0 min, total 26 fractions) were collected. The gradient program for the second fractionation was 20% B from 0 to 7.5 min, 20–100% B linear from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. Fractionation of the eluate was the same as the first HPLC program. In the third HPLC fractionation, the eluant B was replaced by methanol and eluted with 40–65% B linear gradient from 0 to 12.5 min and 65–100% B linear gradient from 12.5 to 17.5 min. Fractions eluted from 2.2 to 17.5 min (total 26 fractions) were collected. In all experiments, suppressive activity of each fraction against HCV RNA expression was measured using replicon cells.

HCV Replicon Cells and Replicon Assay—The Huh-7/3-1 cell line carrying an HCV-replicon was used (10). The line was established from Huh-7 cells by stable transfection with subgenomic selectable RNA in which the encoding HCV structural proteins were replaced by the firefly luciferase gene, the internal ribosome entry site (IRES) of the *Encephalomyocarditis* virus and the neomycin phosphotransferase gene. With this HCV subgenome, the efficiency of subgenomic HCV expression could be estimated by measuring luciferase activity in the replicon cells. The HCV replicon cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with Glutamax (Invitrogen), 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), and 500 μg/ml G418 (Invitrogen). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For the HCV subgenome expression assay, the replicon cells in Dulbecco's modified Eagle's medium supplemented with Glutamax and 5% fetal bovine serum were seeded in 96-well plates (5000 cells/well) and incubated for 24 h. Then the cells were cultured with various concentrations of samples for 72 h. Quantification of the luciferase activity was performed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions, and the luminescence was measured by DTX 800 Multimode Detector (Beckman Coulter, Fullerton, CA). The inhibitory activity was expressed as the concentration required for 50% inhibition (IC_{50}). Specific activity was calculated as a reciprocal number of IC_{50} ($1/IC_{50}$). Total activity was calculated by multiplying yielded weight by specific activity.

The cytotoxicity of the samples was measured by Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 10 μl/well of Cell Counting Kit-8 reagent was added to the cells cultured in a 96-well plate, incubated at 37 °C for 60 min. The absorbance of each well was measured at 450 nm with a reference wavelength at 650 nm using an Emax Precision microplate reader (Molecular Devices Inc., Sunnyvale, CA). Cell viability was calculated as relative index of control cells, and effects of samples on cell viability were expressed as the concentration required for 50% cytotoxicity (CC_{50}).

Constitutive Analysis of Electron Probe Micro-analysis and Liquid Chromatography/Mass Spectrometry-ion Trap-time of Flight (LC/MS-IT-TOF)—For electron probe micro-analysis (EPMA-1600, Shimadzu), the excitation voltage and the beam current were kept at 15 kV and at 100 nA, respectively. The diameter of the electron beam was 50 μm, and the sample was processed for carbon shadowing in advance.

Identification of the anti-HCV compound purified from blueberry leaves was done by HPLC-MSn fragmentation analyses. An HPLC System (Prominence System, Shimadzu) on a reversed-phase column (Atlantis T3, 2.1-mm inner diameter × 100 mm, 3 μm, Waters) was equipped with a photodiode array (PDA) detector scanning from 200 to 800 nm and mass spectrometry-ion trap-time of flight (MS-IT-TOF, Shimadzu)

detector. The mobile phase consisted of a gradient system 30 min of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) at a flow rate of 0.25 ml/min. The elution program was 10–25% B linear from 0 to 7.5 min, 25–100% B linear from 7.5 to 12.5 min, followed by washing 100% B from 12.5 to 20 min. The column was maintained at 40 °C. Electrospray ionization conditions were recorded from m/z = 200 to 1500 in a negative ionization mode. Other MS conditions were as follows: interface voltage, −3.5 or −3.0 kV; nebulizer N_2 gas, 1.5 or 2.0 liters/min; drying N_2 pressure, 200 or 70 kPa, respectively. Heat block temperature and curved desolvation line temperature were both 200 °C. Analytical conditions were recorded from m/z 250 to 1500 in a negative ionization mode. Atmospheric pressure chemical ionization (APCI) probe temperature was set from 250 to 450 °C.

Analysis of Proanthocyanidin—Proanthocyanidins were characterized by a modified method of Porter *et al.* (11, 12), in which they were degraded to anthocyanidins by heating under acidic conditions. Briefly, 200 μl of purified compound from blueberry leaves (0.1–2.5 mg/ml) was mixed with 750 μl of *n*-butanol/HCl (95:5) and 50 μl of 1% of $NH_4Fe(SO_4)_2{\cdot}12H_2O$ dissolved in 2 M HCl. The mixture was vortexed and heated in an oven at 105 °C for 40 min, and cooled in flowing water. Optical densities of the treated solution were recorded at 540 nm by spectrophotometer (UV-1700, Shimadzu). Procyanidin B2 (Sigma-Aldrich) was used as a standard. The hydrolysates generated by the modified Porter method were also analyzed using LC/MS-IT-TOF as described above. The elution program was 10–40% B linear from 0 to 15 min followed by washing 100% B from 15 to 22.5 min. Electrospray ionization conditions were recorded from m/z 200 to 1500 in a positive ionization mode. Interface voltage and nebulizer N_2 were 4.5 kV and 1.5 L/min, respectively. MS/MS conditions were set to auto system and recorded from m/z 50 to 1000. The parent MS was searched from m/z 200 to 1500, and ion accumulation was 30 ms. The data were analyzed by LCMS solution v3.41 software and Formula Predictor Software (Shimadzu).

Thiolysis Analysis—Thiolysis was performed by a previously described method (13, 14) with some modifications. Briefly, 50 μl of purified samples (2 mg/ml in methanol) was mixed with 50 μl of methanol acidified with HCl (3.3%) and 100 μl of benzyl mercaptan (5% in methanol). The reaction was carried out at 50 °C for 30 min and then kept at ambient temperature for 3 h. Pure catechin or epicatechin solution (1.25 mg/ml in methanol) (Funakoshi, Tokyo, Japan) was also thiolyzed to obtain the epimerization rate to calculate the ratio of catechin and epicatechin in the terminal units. The reaction mixture was diluted 5-fold with methanol and analyzed by reverse-phase HPLC. An Atlantis T3 column (4.6 mm × 150 mm, 3 μm, Waters) was used at 40 °C as described above. UV detection was performed at 280 nm. The gradient program was 15–25% B linear from 0 to 10 min, 25–100% B linear from 10 to 30 min, followed by washing 100% B from 30 to 37.5 min and re-equilibration of the column 37.5 to 45 min under initial gradient conditions. To ascertain the elution pattern of thiolysis media and to estimate unknown peaks, LC/MS-IT-TOF was also employed in a negative ion mode. Flavan-3-ols and their benzylthio adducts obtained by thiolysis media of procyanidin B2 were used as a standard. The mDP was calculated by the formula, mDP = [sum of (benzylthio adducts × n) + sum of (free flavan-3-ol × n)]/[total free flavan-3-ol], in which "n" is DP of detected flavan-3-ol by thiolysis.

Preparation of Proanthocyanidin from Blueberry Leaves—To prepare proanthocyanidin from blueberry leaves, freeze-dried powder (100 g) was extracted with 1.2 liters of acetone for 10 min, and the supernatant was decanted. This procedure was repeated five times to remove the green pigment from the leaves, followed by washing in 1.2 liters of hexane for 30 min. The remaining residues were washed with ethyl acetate. The washed powder of leaves was extracted with 1.2 liters of methanol for 30 min, and the supernatant was filtered. This procedure was repeated four times, and the resulting crude methanol extracts were concentrated by rotary evaporator at 50 °C and lyophilized, finally resulting in ~30 g of solid powder. The crude methanol extract (15 g) was then dissolved in 1.0 liter of 60% methanol and placed on a Sephadex LH-20 column (50 mm × 920 mm, Amersham Biosciences). Fractionation was performed using the following series of solvents: fraction I, 9.0 liters of 60% methanol (retrieved weight: 10.2 g); fraction II, 9.0 liters of 100% methanol (retrieved weight: 3.3 g); fraction III, 9.0 liters of 70% (v/v) acetone (retrieved weight: 1.3 g). The LC/MS-IT-TOF analyses of each fraction indicated that fraction I was primarily composed of quinic acid, chlorogenic acid, and flavonol glycosides such as rutin. Fraction II consisted of proanthocyanidin oligomers from tetramer to decamer as analyzed by thiolysis. Fraction III consisted of proanthocyanidin polymers that were decamers or greater. In each fraction, the eluate was divided into 28 subfractions/liter.

Northern Blot Analysis—Total RNAs from cultured replicon cells were prepared using RNeasy mini kits (Qiagen). RNAs were denatured at 65 °C for 15 min, cooled on ice, and then separated by 1% agarose-formaldehyde gel electrophoresis (2 μg/lane) and transferred to a positively charged nylon membrane (Hybond-N^+, Amersham Biosciences). The membrane was hybridized with a biotinized probe of the neomycin phosphotransferase gene. For detection of the bound probe, membranes were incubated with streptavidin-Alexa Fluor 680 conjugate (Invitrogen), and the bound fluorescence was detected by Odyssey Infrared Imaging System (LI-COR Biosciences). For internal control, β-actin mRNA-specific biotinized antisense RNA probe was used.

Western Blot Analysis—Cultured replicon cells were harvested, and total cellular proteins were extracted with Cel-Lytic-M (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). The samples were separated by SDS-PAGE using 10% gel under reducing conditions. The proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA).

The membrane was treated with a blocking buffer for near infrared fluorescent Western blotting (Rockland, Gilbertsville, PA). Primary antibodies used were anti-human hnRNP A2/B1, hnRNP K, hnRNP L, and hnRNP Q and anti-human β-actin antibodies (EF-67, D-6, A-11, 18E4, and I-19, respectively, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human eukaryotic translation initiation factor 3 (eIF3) F, eIF3G eIF3H polyclonal antibodies (Novus Biologicals, Littleton, CO), and

anti-HCV NS-3 polyclonal antibody (10). The labeled proteins were visualized with Alexa Fluor 680 anti-rabbit or anti-mouse IgG (Invitrogen) or IRDye™ 800CW anti-goat IgG (LI-COR Biosciences) and detected by using as Odyssey Infrared Imaging System.

Affinity Purification of Proanthocyanidin-binding Proteins—Purified blueberry leaf-derived proanthocyanidin or catechin was coupled with epoxy-activated Sepharose 6B (Amersham Biosciences) according to the manufacturer's instructions. Approximately 5×10^8 HCV replicon cells were extracted with lysis buffer (50 mM sodium phosphate (pH 7.5), 1% CHAPS, 5 mM EDTA, 150 mM NaCl, and protease inhibitor mixture (Complete™, Roche Diagnostics, Mannheim, Germany)). The total protein extract (90 mg) was added to the coupled Sepharose beads (3 ml) and incubated at 4 °C overnight with gentle rotation. The beads were centrifuged ($500 \times g$) for 1 min, and the pellet was washed six times with the lysis buffer. The absorbed proteins were eluted by incubation in 2% SDS with 50 mM dithiothreitol at 100 °C for 10 min. The eluate was concentrated with an Amicon Ultra-4 Ultracel-5k (Millipore), and the solvent was replaced by the lysis buffer. Protein concentration was determined by the *o*-phthalaldehyde method using bovine serum albumin as the standard.

Fluorescent Two-dimensional DIGE—Fluorescent two-dimensional-DIGE was performed using fluorescent dyes, IC3-OSu and IC5-OSu (Dojindo Molecular Technologies), with a modification of the methods reported elsewhere (15, 16). Briefly, 10 μg of proteins per gel were precipitated using a two-dimensional clean-up kit (Bio-Rad) and then dissolved in 20 μl of sample buffer (10 mM sodium phosphate (pH 8.0), 7 M urea, 2 M thiourea, 3% CHAPS, and 1% Triton X-100). After addition of 400 pmol of IC3-OSu or IC5-OSu, proteins were incubated at 40 °C for 30 min. The labeling reaction was quenched by incubation with 400 μM lysine for 15 min, followed by addition of an equal volume of the sample buffer with 150 mM dithiothreitol, 0.4% Bio-Lyte 3–10 (Bio-Rad Laboratories), and 0.004% bromphenol blue. Two-dimensional gel electrophoresis was performed according to the manufacturer's instructions (Bio-Rad). The mixed samples were applied to ReadyStrip IPG strips (pH 3–10 NL, 7 cm, Bio-Rad) for separation in the first dimension. The second-dimensional separation was performed by SDS-PAGE using an 8% gel. Fluorescence imaging was performed on a Proxpress™ proteomic imaging system (PerkinElmer Life Sciences). IC3-OSu-labeled proteins were detected with 540/25 nm excitation and 590/35 nm emission filters. IC5-OSu-labeled proteins were detected with 625/35 nm excitation and 680/30 nm emission filters. In this study, while proteins from proanthocyanidin- or catechin-coupled Sepharose were labeled with IC5-OSu, a mixture of equal quantities of both samples was labeled with IC3-OSu and used as a reference for quantitation of IC5-OSu-labeled proteins as described (16). The fluorescent images were aligned using SameSpot TT900 S2S (Nonlinear Dynamics, Newcastle, UK) and then analyzed with Progenesis Discovery software (Nonlinear Dynamics). Each group (eluate from proanthocyanidin- or catechin-coupled Sepharose) was run on triplicate gels three times. Spot intensity in the IC5-OSu image was normalized to the intensity of the corresponding IC3-OSu image spot in the same gel. The average spot intensities ± standard deviation (S.D.) from nine gels were calculated. Statistical differences were determined by Student's *t* test, and *p* values <0.05 were considered significant. The proteins having high affinity to proanthocyanidin but not to catechin were detected using a 1.5-fold change ($p < 0.05$) as the cut off.

Protein Identification—Protein identification by peptide mass fingerprinting was performed as described previously (17). Briefly, 100 μg of proteins was separated by two-dimensional-DIGE gels and stained with Coomassie Brilliant Blue R-250. Protein spots of interest were excised from the gel and digested overnight with trypsin. Each peptide extract was deposited onto a thin layer of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) and allowed to adsorb for 5 min, after which the layers were washed twice with 0.1% trifluoroacetic acid. Spectra were obtained using matrix-assisted laser desorption/ionization-TOF-TOF-MS, Autoflex II TOF/TOF (Bruker Daltonics) in positive-ion and reflectron mode. The data set was entered in an in-house Mascot search engine (Matrix Science, London, UK), to find the closest match with known proteins registered in the data base from the Swiss-Prot.

Knockdown of Proanthocyanidin-binding Proteins Using siRNAs—ON-TARGETplus SMARTpools of duplex siRNAs targeting hnRNP L, hnRNP K, hnRNP A2/B1, hnRNP A/B, hnRNP Q, eIF3F, eIF3G, eIF3H, and non-targeting control siRNA were purchased from Dharmacon (Thermo Fisher Scientific, Tokyo, Japan). Individual sequence of hnRNP A2/B1 siRNAs was confirmed by two single siRNAs (Target #09: 5′-CGGUGGAAAUUUCGGACCA-3′, Target #11: 5′-GGAGAGUAGUUGAGCCAAA-3′). The replicon cells were transfected with each siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. After 72 h incubation, the cells were assayed.

RESULTS

Purification of an Inhibitor of HCV Subgenome Expression from Blueberry Leaves—We screened 283 species of local agricultural products for their suppressive activity against the expression of subgenomic HCV RNA using an HCV replicon cell system, and found significant suppressive activity in the leaves of the blueberry (*Vaccinium virgatum* Aiton), peels of roots of Taro (*Colocasia esculenta* L.), and hulls of seeds of Japanese plum (*Prunus mume* Sieb. *et* Zucc). Among them, extracts of blueberry leaves contained the highest total activities. Therefore, we purified a compound from blueberry leaves that inhibited expression of subgenomic HCV RNA in replicon cells. An overall purification scheme is shown in Fig. 1, and a summary of the purification steps is shown in Table 1. From 1000 mg of lyophilized powder from the leaves, 440 mg of methanol extracts was obtained. The IC_{50} value of the methanol extracts was 5.47 μg/ml. The inhibitory activity was recovered in the CMW-W fraction (284.2 mg), in which the IC_{50} value was 1.74 μg/ml. The specific activity of CMW-W was 3-fold greater than that of the initial methanol extracts and the yield of the activity exceeded 200%, suggesting that an interfering substance had been removed.

The CMW-W fraction was subjected to a subsequent HPLC purification step in which a preliminary HPLC elution pattern

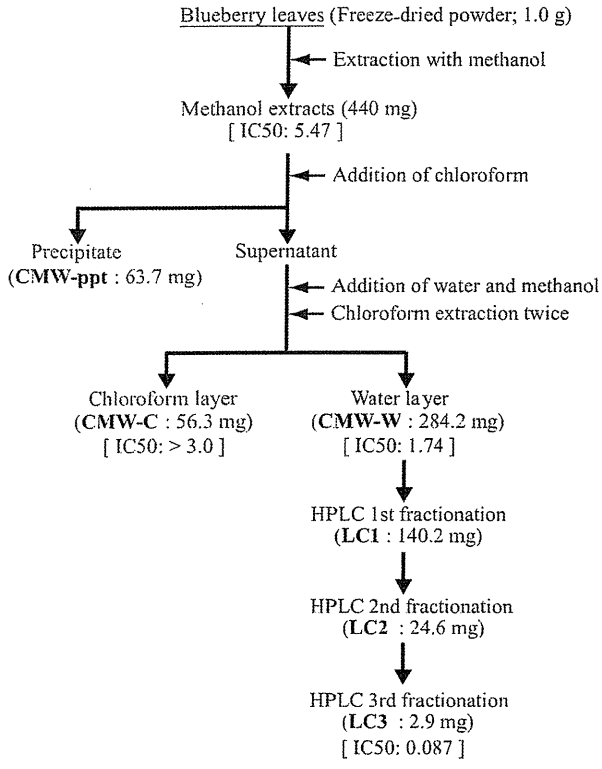

FIGURE 1. **Fractionation of blueberry leaf extract for the inhibitor of HCV subgenome expression.** The inhibitory activity was indicated under each fraction as the IC_{50} value (micrograms/ml).

TABLE 1
Purification of HCV subgenome expression inhibitory activity in blueberry leaf

	Total weight	Subgenome expression, IC_{50}	Specific activity	Purification factor	Total activity	Yield
	mg	*μg/ml*	$1/IC_{50}$		mg/IC_{50}	%
MeOH extract	440.0	5.47	0.18	1.00	80.44	100
Water layer	284.2	1.74	0.57	3.14	163.33	203.05
LC 1st	140.2	0.89	1.12	6.15	157.53	195.84
LC 2nd	24.6	0.54	1.85	10.13	45.56	56.63
LC 3rd	2.9	0.087	11.49	62.87	33.33	41.44

(a 15–100% gradient of acetonitrile) was used. The data indicated that a strong inhibitory activity eluted around 90% of acetonitrile (17 min) with some minor inhibitory activities broadly eluted earlier. Those results suggested the possible existence of multiple HCV subgenome expression inhibitors in the CMW-W fraction (Fig. 2*A*). To purify the most active component, we initially separated the CMW-W isocratic at 30% acetonitrile and collected the active fraction eluted at 3.3–5.2 min (Fig. 2*B*). After repeated collection, we obtained 140.2 mg of active fraction (LC1) from 440 mg of methanol extracts. The IC_{50} value of this fraction for HCV RNA expression was 0.89 μg/ml, yielding a specific activity 6-fold higher than that of the initial methanol extracts (Table 1). In the second round HPLC (Fig. 2*C*), we fractioned LC1 as follows: 20% acetonitrile from 0 to 7.5 min, followed by 20–100% linear gradient of acetonitrile from 7.5 to 12.5 min. A highly active fraction was eluted from 11.9 to 13.2 min and collected (LC2), yielding 24.6 mg with an IC_{50} value 0.54 μg/ml (Table 1). In the third HPLC step (Fig. 2*D*), we applied LC2 and eluted with 40–65% methanol instead of acetonitrile. The active fraction was eluted from 3.2 to 6.2 min and collected (LC3), finally yielding 2.9 mg of solid material with a dark flesh color. The IC_{50} value for HCV RNA expression of LC3 was 0.087 μg/ml, with a 63-fold increase in specific activity relative to the initial methanol extracts (Table 1). We also checked the cytotoxic effect on replicon cells. The CC_{50} value of the cytotoxicity of LC3 was 18.5 μg/ml, and the selective index, which was calculated by dividing CC_{50} by IC_{50}, was 212.6, showing a 16.5-fold higher selective index value compared with initial methanol extracts (Fig. 3).

The Inhibitor of HCV Subgenome Expression Is Proanthocyanidin—To analyze the constituent elements in the purified fraction LC3, EPMA was performed. This analysis indicated that the fraction is composed of carbon and oxygen, but not nitrogen (data not shown). In addition, trace amounts of calcium, sodium, potassium, and aluminum, which appeared to be contaminating elements, were also identified. Next, LC3 was analyzed by LC/MS-IT-TOF. Preliminary trials showed that analysis required the use of an APCI probe at 450 °C, and no signal was obtained at 250 °C. The mass spectrum data showed five peaks (Fig. 4), and $[M-H]^-$ at *m/z* 401.0494 and 689.1135 were considered to be trifluoroacetic acid adducts of *m/z* 287.0553 and 575.1196, respectively. From these spectra, the parent mass of this compound appeared to be $[M-H]^-$ at *m/z* 575.1196, which was estimated to be $C_{30}H_{24}O_{12}$ (error = 0.17 ppm), an A-type dimer of procyanidin. Given the fact that strict conditions (APCI probe temperature at 450 °C) were required to ionize the compound, it appeared that the isolate consisted of one or more polymers of procyanidin.

We next analyzed the purified LC3 fraction by butanol-HCl hydrolysis (Porter method) (11, 12). The reacted solution turned a red color, which is in accordance with the color of anthocyanidin generated by heating of procyanidin/proanthocyanidin under acidic condition. Using procyanidin B2 as a standard, the procyanidin content in the LC3 fraction was 86.33%. The hydrolysis solution was analyzed by LC/MS-IT-TOF. The main peak (retention time = 7.3 min) of the PDA chromatogram at 540 nm was observed at the same position as that of the cyanidin standard (Fig. 5*A*). Indeed, MS/MS spectra of this peak were identical to those of the cyanidin standard (Fig. 5*B*). These results revealed that the HCV RNA replication inhibitory compound present in the LC3 fraction from blueberry leaves was procyanidin. Because the hydrolysate of this compound also contained a trace amount of delphinidin (Fig. 5*A*, *arrow*), this compound was considered to be proanthocyanidin rather than procyanidin.

Structural Analysis of the Inhibitory Proanthocyanidin by Thiolysis—To analyze the terminal and extension units and also define mDP of proanthocyanidin in the purified LC3 fraction of blueberry leaves, we combined thiolysis (13) with reversed-phase HPLC. When thiolysis products of purified proanthocyanidin in the LC3 fraction were analyzed in reversed-phase HPLC, several peaks (*A–H*) were identified (Fig. 6). The peaks *A*, *C*, and *H* were considered to be catechin, epicatechin, and benzylmercaptan, respectively, according to the retention time of each standard preparation. Other peaks were confirmed by analyzing mass spectra. The parent mass of peak E was $[M-H]^-$

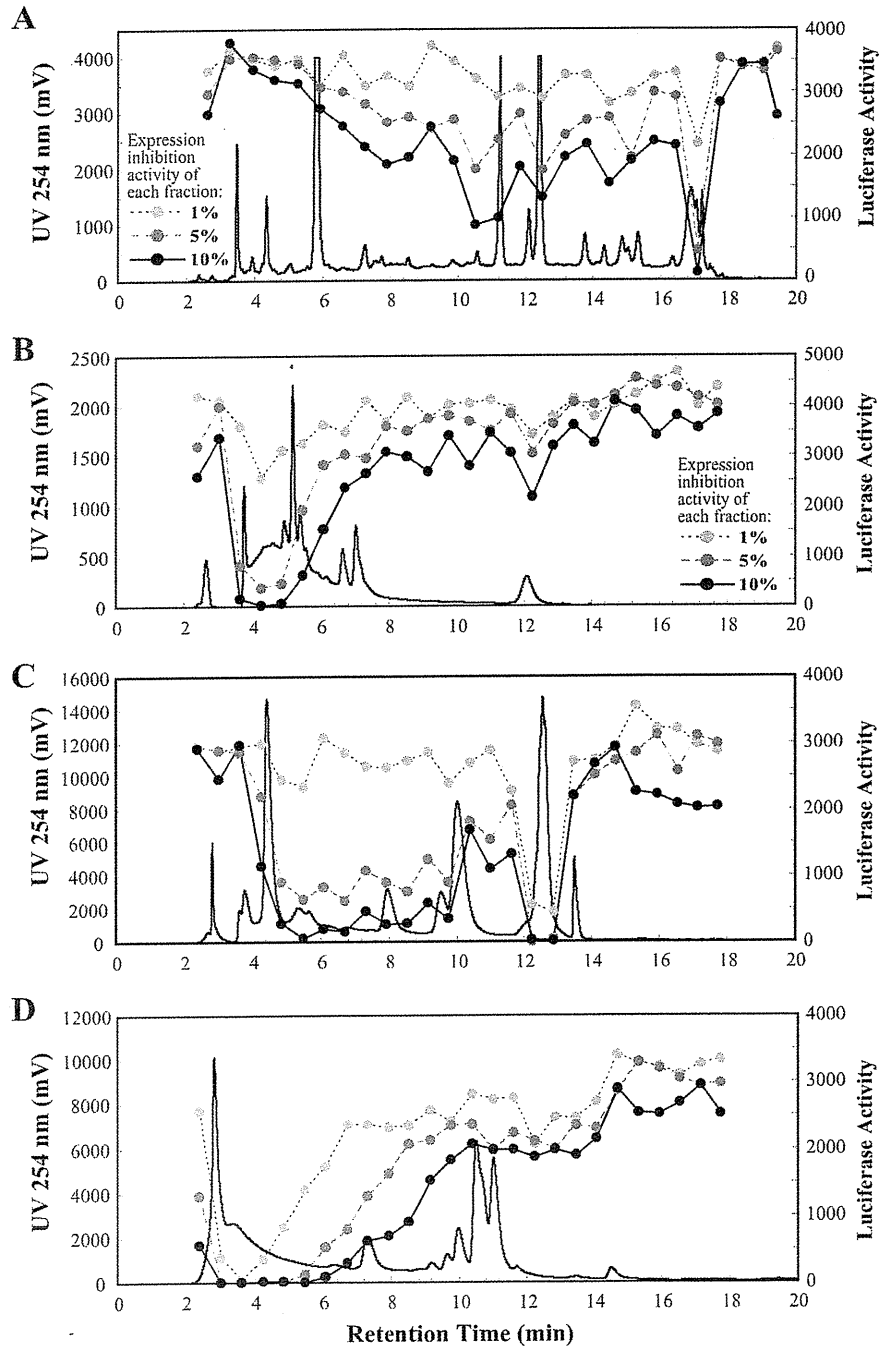

FIGURE 2. **HPLC chromatogram and HCV subgenome expression-inhibitor activity.** Replicon assays were performed using three different concentrations (1%, 5%, and 10%) of each eluted fraction and are indicated as luciferase activity. The elution conditions are indicated under "Experimental Procedures". *A*, preliminary HPLC chromatogram and suppressive activities against HCV subgenome expression in replicon cells. The applied sample was eluted with a 15–100% gradient of acetonitrile. *B*, first preparative fractionation (*LC 1st fractionation*). CMW-W fraction was applied, and the eluate was collected from 2.1 min to 18 min (445 μl/fraction, total 26 fractions). Fractions with significant inhibitory activity, eluted from 3.3 to 5.2 min, were collected. *C*, second preparative fractionation (*LC 2nd fractionation*). The collected sample from the first LC fractionation was subsequently separated by HPLC, and fractions with significant inhibitory activity eluted from 11.9 to 13.2 min were collected. *D*, third preparative fractionation (*LC 3rd fractionation*). Sample collected in the second LC fractionation was further separated by HPLC, and fractions with significant inhibitory activity, eluted from 3.2 to 6.2 min, were collected.

at m/z 411.0892, with an estimated formula of $C_{22}H_{20}O_6S$ (error = −3.8 ppm), and its MS/MS spectrum was $[M-H]^-$ at m/z 287.0510. The difference between the parental mass and MS/MS was 124.0382, which was in accordance with a benzylthio adduct. Thus, peak E appeared to be catechin or epicatechin benzylthioether. Because the retention time of epicatechin benzylthioether was the same as that of peak E, we considered peak E to be epicatechin benzylthioether. The parental mass of peak G was $[M-H]^-$ at m/z 697.1385 (predicted formula: $C_{37}H_{30}O_{12}S$), and its MS/MS was $[M-H]^-$ at m/z 573.0987. Again, the difference was 124.0398 and likely represented the benzylthio adduct. Thus, peak G was estimated to be a benzylthioether of A-type dimer consisting of catechin and/or epicatechin. Peak B was detected as parent MS $[M-H]^-$ at m/z 863.1822 with a predicted formula $C_{45}H_{36}O_{18}$ (error = −0.86 ppm). Because the formula of B-type procyanidin trimer is $C_{45}H_{38}O_{18}$ and that of A-type is $C_{45}H_{34}O_{18}$, this peak was likely a trimer in which A-type and B-type interflavan bonds coexisted. Peak D was suggested to be an A-B type trimer similar to peak B but with a benzylthio adduct. The parental mass of peak F was $[M-H]^-$ at m/z 605.1449, and its MS/MS was $[M-H]^-$ at m/z 481.1109, so that a benzylthio adduct was also present in peak F. However, we could not obtain the predicted formula of the parental mass of peak F. The structural analysis of the HCV inhibitor proanthocyanidin from blueberry leaves (fraction LC3) is summarized in Table 2. The mDP of proanthocyanidin in this fraction was estimated to be 7.7. Because the predicted formula of peak F was undefined, peak F is indicated as "unknown" in Table 2.

Role of Polymerized Structure of Proanthocyanidin in the Inhibition of HCV Subgenome Expression—Because the purified HCV expression-inhibitory proanthocyanidin of blueberry leaf was oligomer with mDP 7.7, we asked whether the polymerization was required for inhibitory activity. First, the inhibitory activities of monomers such as catechin, epicatechin, and epigallocatechin-gallate, all of which were constituents of proanthocyanidin, and also of the dimer (procyanidin B2) were

tested by HCV replicon assay. These monomers and the dimer of procyanidin lacked inhibitory activity (Table 3).

We then determined how the degree of polymerization of proanthocyanidin affected the inhibition. The crude fraction of proanthocyanidins was obtained by the extraction of three low polarity solvents (acetone-hexane-ethyl acetate) as described under "Experimental Procedures." The IC_{50} of HCV RNA expression of this proanthocyanidin-enriched fraction was 3.20 μg/ml, showing greater activity than the crude methanol extract. After fractionation on a Sephadex LH-20 column, each eluant was analyzed by LC/MS-IT-TOF and thiolysis to determine the components and mDP of proanthocyanidin (supplemental Fig. S1). Then, the blueberry leaf-derived proanthocyanidins with different mDP were assessed for HCV inhibitory activity. The inhibitory activity of blueberry leaf proanthocyanidin was clearly dependent on the polymerization level, and the peak activity was observed at a polymerization level of ~8 to 9 (IC_{50}: 0.05 μg/ml) (Fig. 7).

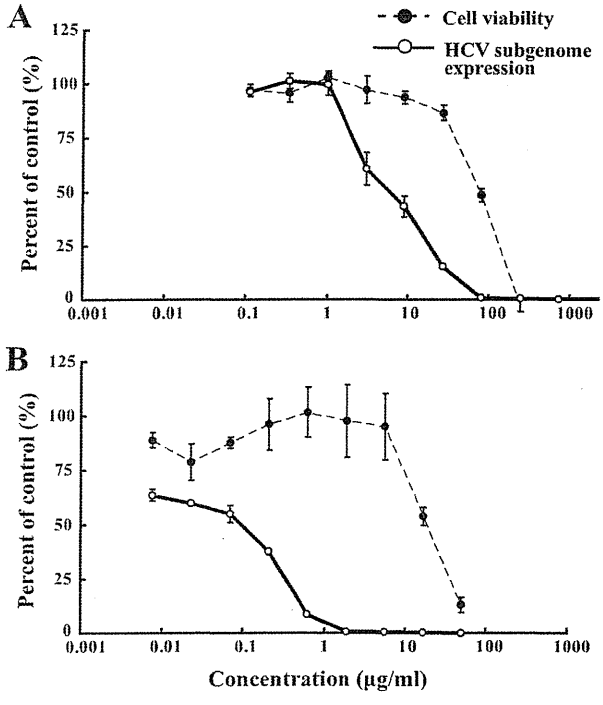

FIGURE 3. **Dose-dependent effects of blueberry-derived samples on subgenomic HCV RNA-expression inhibition and viability of replicon cells.** *A*, dose-dependent effects of methanol extracts of blueberry leaves. Concentrations of the sample from 0.112–2200 μg/ml were tested. IC_{50} for HCV expression and CC_{50} for cytotoxicity were 5.47 μg/ml and 70.61 μg/ml, respectively, and the selective index was 12.9. *B*, dose-dependent effects of purified sample (LC 3rd fractionation). Concentrations of the sample from 0.01 to 50 μg/ml were tested. The IC_{50} values for HCV subgenome expression and cytotoxicity were 0.087 μg/ml and 18.50 μg/ml, respectively, and the selective index was 212.6.

Effect of Purified Blueberry Proanthocyanidin on the Expression of NS3 HCV Protein in Replicon Cells—In our system, HCV RNA expression in replicon cells was expressed as luciferase activity. Thus, the observed inhibitory activity may have resulted from nonspecific inhibition of luciferase by proanthocyanidin. Therefore, we examined the effect of the purified proanthocyanidin (fraction LC3) on the expression levels of the neomycin-resistant gene and the NS3 protein gene, both of which were encoded in the HCV subgenome of replicon cells. The purified blueberry proanthocyanidin suppressed the expression of the neomycin-resistant gene and also the levels of NS3 protein in a concentration-dependent manner, indicating that the proanthocyanidin purified from blueberry leaves in fact suppressed the expression of HCV subgenome in the replicon cells (Fig. 8).

hnRNP A2/B1, Which Has Affinity to Proanthocyanidin, Is Indispensable for Expression of Subgenomic HCV RNA—To investigate the molecular mechanism underlying the suppression of HCV RNA expression by proanthocyanidin, we comprehensively identified proteins having affinity to the purified proanthocyanidin from blueberry leaves. The protein extract from replicon cells was treated with proanthocyanidin-coupled Sepharose, and then the adsorbed proteins were eluted. The extract was also treated with Sepharose beads coupled to catechin, a structural unit of proanthocyanidin, but HCV subgenome-expression inhibitory activity was not observed (Table 3). The proteins having higher affinity to proanthocyanidin than catechin were detected with fluorescent two-dimensional-DIGE (Fig. 9). In the eluate from proanthocyanidin-coupled Sepharose, intensities of 32 spots were increased compared with those from catechin-coupled Sepharose. Twenty-seven spots were cut from Coomassie-stained gels and subjected to peptide mass fingerprinting using MS, and we successfully identified proteins derived from 25 spots (Nos. 1 to 25 in Fig. 9*A* and Table 4). Although other possible candidate spots were also suggested in a rectangular portion (Fig. 9*A*), they were not subjected to protein identification due to insufficient separation.

From the list of identified proteins (Table 4), most could be categorized into two groups. The first group consisted of subunits of eukaryotic translation initiation factor 3 (eIF3). They included eIF3A (spot Nos. 1, 5, and 9), eIF3F (No. 10), eIF3G (No. 12), eIF3H (No. 4), and eIF3M (No. 13). Although eIF3A was identified from multiple protein spots (Nos. 1, 5, and 9), this may be due to post-translational modification and protein processing. The second group of proteins consisted of hnRNPs such as hnRNP A/B (No. 19), hnRNP A2/B1

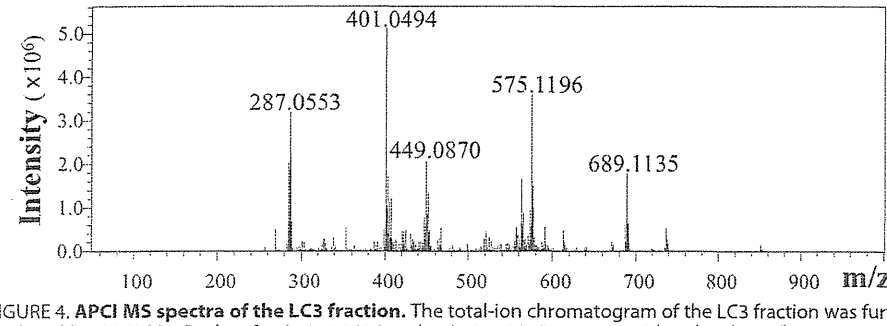

FIGURE 4. **APCI MS spectra of the LC3 fraction.** The total-ion chromatogram of the LC3 fraction was further analyzed by APCI MS. Peaks of *m/z* 401.0494 and *m/z* 689.1135 were considered to be trifluoroacetic acid adducts of *m/z* 287.0553 and *m/z* 575.1196, respectively. Parental MS of this compound was estimated at *m/z* 575.1196, and the formula was assumed to be $C_{30}H_{24}O_{12}$.

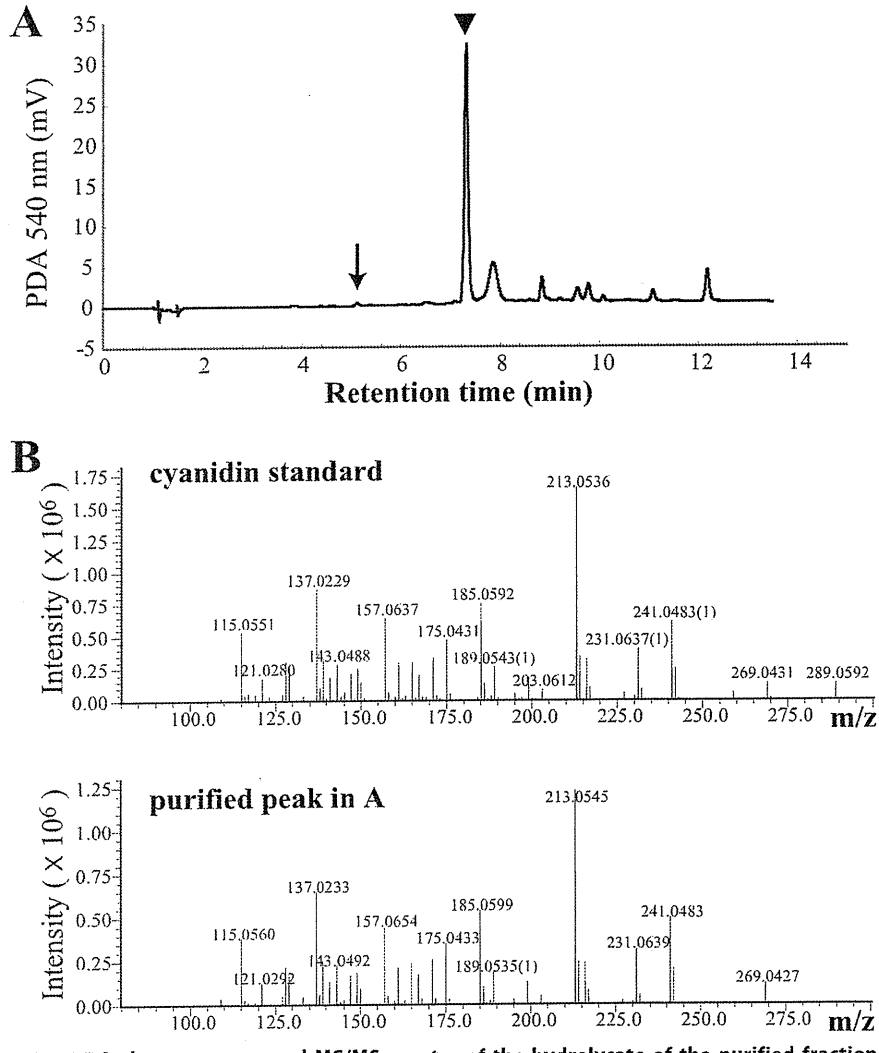

FIGURE 5. **PDA chromatogram and MS/MS spectra of the hydrolysate of the purified fraction by the Porter method.** *A*, PDA chromatogram at 540 nm of hydrolysate of purified fraction from blueberry leaves. The main peak (*arrowhead*; retention time = 7.3 min) is located at the same position as the cyanidin standard. Other peaks were estimated to be methoxylated cyanidins from MS and MS/MS spectra. The *arrow* indicates the position of delphinidin. *B*, MS/MS spectra (positive ion mode) of hydrolysate of cyaniding standard (*upper panel*; parent MS at 287.0550) and the purified peak in A (*lower panel*; parent MS at 287.0555).

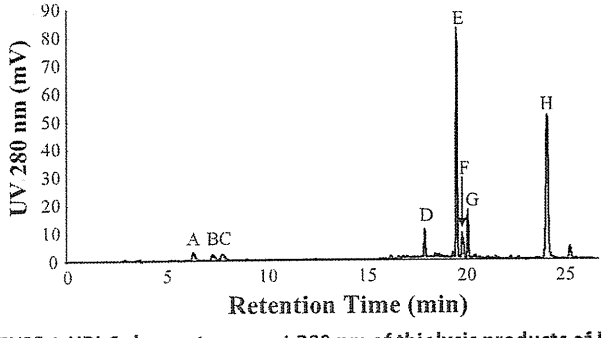

FIGURE 6. **HPLC chromatogram at 280 nm of thiolysis products of LC3 fraction from blueberry leaves.** *Peaks A*, *C*, and *H* were identified as catechin, epicatechin, and benzylmercaptan, respectively. *Peak E* was identified as epicatechin benzylthioether. *Peaks B* and *D* were estimated to be procyanidin trimer with coexistence of A-type and B-type linkages and its benzylthioether, respectively. *Peak G* was procyanidin A-type dimer. *Peak F* was not identified.

(No. 8), hnRNP K (Nos. 17 and 22), hnRNP L (Nos. 11, 15, and 21), and hnRNP Q (Nos. 2, 6, and 7) also known as NS1-associated protein 1. Importantly, eIF3 has been reported to bind directly to the HCV internal ribosome entry site (IRES), leading to translation initiation of viral proteins (18). Moreover, all hnRNPs identified have been reported to be associated with HCV genomic RNA such as IRES and non-translated regions (19–25). These results imply that proanthocyanidin may target cellular proteins such as eIF3 and hnRNPs. To further clarify the relationship between these proteins and HCV subgenome expression, we examined the effects of siRNA-based knockdown of these proteins (supplemental Fig. S2). First, we selected three eIF3 subunits (eIF3F, eIF3G, and eIF3H), which are thought to be involved in IRES binding of eIF3 (26). However, knockdown of these subunits did not affect the luciferase activity in replicon cells. Then, we targeted all hnRNPs identified. Among them, siRNA pool targeting hnRNP A2/B1 significantly suppressed the luciferase activity of HCV subgenomic replicon cells (supplemental Fig. S2), and this result was confirmed using two kinds of single siRNA (Fig. 10). Weak suppressive activities were also suggested by siRNAs targeting other hnRNPs such as hnRNP A/B, K, and L (supplemental Fig. S2).

DISCUSSION

The HCV infection is a major cause of chronic liver disease, which eventually results in end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. A crude extract from rabbit-eye blueberry (*V. virgatum* Aiton) leaves exhibited significant inhibitory activity against HCV RNA expression when analyzed in HCV subgenomic replicon cells. In this study, we attempted to purify a compound that suppresses HCV subgenome expression from the blueberry leaves. The final purified product was identified as proanthocyanidin, and it was effective at concentrations that are two orders of magnitude below the toxic threshold in replicon cells. The mDP of the proanthocyanidin in purified anti-HCV expression fraction was 7.7 with a high proportion of epicatechin as the monomeric components. Subsequent analysis indicated that the blueberry leaf-derived proanthocyanidin with a degree of polymerization of ~8–9 shows the highest inhibitory activity. Finally, the purified pro-

TABLE 2
Thiolysis results of purified fraction (LC3) from blueberry leaves

	mDP	Terminal				Extension					
		C[a]	EC[b]	AB-3[c]	Total	C[a]	EC[b]	A-2[d]	Unknown	AB-3[c]	Total
						%					
LC3	7.7	20.4	65.1	14.5	100	0.8	58.1	11.9	23.2	6.0	100

[a] Catechin.
[b] Epicatechin.
[c] Trimer consisting of both A-type and B-type interflavan bonds.
[d] A-type dimer.

TABLE 3
Effects of constitutional units of proanthocyanidin on expression of HCV subgenome in replicon cells

Compounds	DP or mDP	Subgenome expression, IC_{50}	Cytotoxicity, CC_{50}	Ratio, CC_{50}/IC_{50}
		μg/ml	*μg/ml*	
Catechin	1	16.18	100.4	6.2
Epicatechin	1	27.32	113.8	4.2
Epigallocatechin-gallate	1	14.61	41.68	2.9
Procyanidin B2[a]	2	>25.0	>25.0	–
Purified proanthocyanidin from blueberry leaf (LC3 fraction)	7.7	0.087	18.5	212.0

[a] Epicatechin dimer.

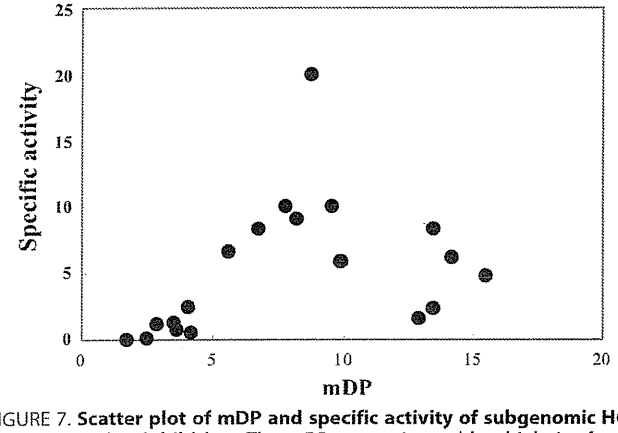

FIGURE 7. **Scatter plot of mDP and specific activity of subgenomic HCV RNA-expression inhibition.** The mDP was estimated by thiolysis of each fraction. The specific activity was calculated from IC_{50} value of each fraction.

anthocyanidin from blueberry leaf extracts suppressed the expression of the neomycin phosphotransferase gene and the NS-3 protein gene in HCV subgenome replicon cells in a dose-dependent manner. These data suggest the potential value of blueberry leaf proanthocyanidin for the treatment of HCV infection.

Proanthocyanidin is a polyphenol that shows polymerization of more than two units of flavan-3-ol such as catechin and epicatechin (supplemental Fig. S3). There are two interflavan bonds in proanthocyanidin, in which the B-type has one linkage of interflavan bond (C4 → C8 or C4 → C6) and the A-type has two linkages of bonds (C4 → C8 and O7 → C2) (27). Proanthocyanidins were previously known as condensed tannin and are present in various plants and foods. They contribute to organoleptic properties such as stability, astringency, and bitterness (28, 29). There are a number of foods and nutritional supplements that contain proanthocyanidins with health-promoting benefits, and their value has been described in the literature and patent documents. For example, proanthocyanidin contained in blueberries increases the lifespan of the nematode (*Caenorhabditis elegans*) (30). Sangre de Grado extracted from *Croton*

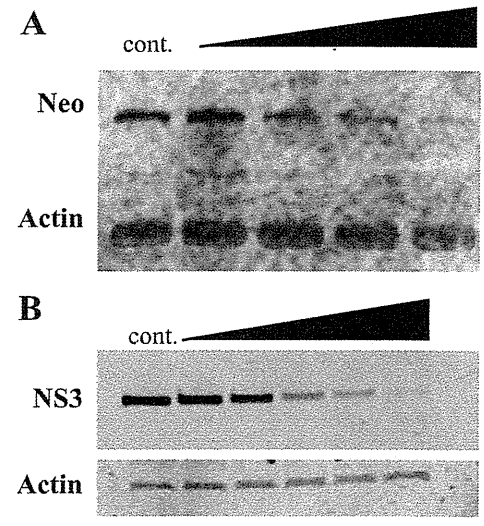

FIGURE 8. **Suppressive effects of purified blueberry leaf proanthocyanidin (LC3 fraction) on the expression of the neomycin resistant gene and NS-3 protein in replicon cells.** *A*, Northern blot analysis of the *neomycin-resistant* gene expression (*Neo*) in the presence of 0 μg/ml (control) to 3.3 μg/ml proanthocyanidin in a 3-fold dilution series. The expression of β-actin mRNA is also indicated as a normalization control. *B*, Western blot analysis of the expression of NS-3 protein (*NS3*) in the presence of 0 μg/ml (control) to 10 μg/ml proanthocyanidin in a 3-fold dilution series. The β-actin protein levels are also shown as a normalization control.

lechleri resin is a traditional natural medicine in the upper Amazon and contains hydrolyzing flavonoids, proanthocyanidins, and other polyphenols (31, 32), which have been shown to possess anti-viral activities against influenza, parainfluenza, herpes simplex viruses, and respiratory syncytial virus (33–38). However, to the best of our knowledge, this report is the first study to demonstrate that proanthocyanidin inhibits the expression of subgenomic HCV RNA.

Regarding the mechanism underlying the anti-viral activities, proanthocyanidins from *Croton lechleri* resin and prodelphinidin B-2 3′-*O*-gallate from green tea leaf inhibit herpes simplex viruses infection by preventing the attachment and penetration of the virus into the target cells (37, 39). Recently, the grapefruit flavonoid naringenin was reported to inhibit apolipoprotein B-dependent HCV secretion (40). However, in this study, we evaluated the inhibitory effect on HCV subgenome expression by measuring luciferase activity in replicon cells without using actual viral particles. Therefore, the mode of anti-HCV action of proanthocyanidin is different from that in herpes simplex viruses infection mentioned above and is also different from the inhibitory mechanism of naringenin. Instead, our study suggests that blueberry leaf-derived proanthocyanidin may interact with hnRNP A2/B1, a factor required for HCV subgenome expression in our replicon assay. In accordance with this observation, recent study has shown that hnRNP A1, a protein highly homologous to hnRNP A2/B1, facilitates HCV replication, and the double knockdown of hnRNP A1 and hnRNP A2 significantly suppresses replication (23). Alternatively, proanthocyanidin may bind to the translational initiation complex associated with HCV IRES and thereby suppresses the HCV subgenome expression, because a number of translational regulatory proteins are included in our list of proanthocyanidin-binding proteins. To date, for the

inhibitors of IRES-directed translation in HCV-infected cells, vitamin B12, a synthetic peptide derived from human La protein, and RNA molecules targeting IRES have been reported (42–44). However, little is known regarding the effect of natural product-derived polyphenolic compounds on HCV IRES-directed translation, and this possibility should be clarified in a future study. It should be noted that all proanthocyanidin-binding proteins identified in this study are intracytoplasmic and/or intranuclear proteins. However, it is not known whether proanthocyanidin can be efficiently translocated into the intracellular space despite its highly polymerized structure. Nonetheless, absorption of proanthocyanidin from the digestive tract has been reported (12, 45), suggesting the possibility of proanthocyanidin internalization into cells, and internalization of high molecular weight molecule via clathrin-mediated endocytosis, caveolae-mediated uptake or pinocytosis has been reported (46). Further studies are in progress, focusing on the intracellular uptake of proanthocyanidin.

The current therapies for hepatitis C patients are based on a combination of pegylated recombinant interferons and ribavirin. However, viral clearance is achieved by <60% of treated patients, and the therapies are limited by significant side effects and high costs (47, 48). Therefore, many novel anti-HCV drugs are currently under development, most of which target viral enzymes. For example, BILN-2061, VX-950, and SCH503034 are inhib-

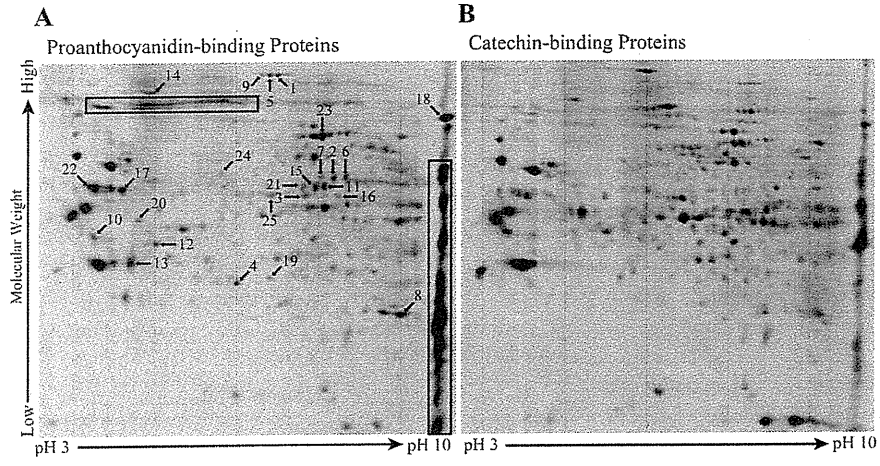

FIGURE 9. **Fluorescent two-dimensional-DIGE images of proteins with affinities to blueberry leaf proanthocyanidin (*A*) and catechin (*B*).** Protein extracts from replicon cells were treated with epoxy-activated Sepharose coupled to proanthocyanidin or catechin. The adsorbed proteins were eluted and then visualized as protein spots using fluorescent two-dimensional-DIGE. Fluorescent intensities were measured using Progenesis Discovery software. In the eluate from proanthocyanidin-coupled Sepharose (*A*), intensities of 32 spots were increased relative to those from catechin-coupled Sepharose (*B*). Twenty-seven spots were subjected to subsequent analysis and proteins derived from 25 spots (*spot nos. 1–25* in *A*) were identified with peptide mass fingerprinting. *Spot numbers* correspond to those in Table 4. Proteins in regions of high molecular weight and high pI (*rectangular regions*) were not sufficiently separated and were not analyzed.

TABLE 4
Proteins with higher affinity to blueberry proanthocyanidin than to catechin

Spot no.[a]	Intensity[b] (×10^5)		Ratio[c]	*p* value[d]	Protein name[e]	Accession number[f]	Coverage[g]	Molecular mass[h]	pI[i]
	Proanthocyanidin	Catechin							
							%	*kDa*	
1	5.69 ± 2.98	0.99 ± 0.24	5.73	0.0015	Eukaryotic translation initiation factor 3 subunit A (eIF3A)	Q14152	12.4	166.9	6.38
2	6.96 ± 1.33	1.39 ± 0.30	5.00	<0.0001	hnRNP Q	O60506	27.0	69.8	8.68
3	5.63 ± 0.71	1.15 ± 0.36	4.88	<0.0001	Splicing factor U2AF 65-kDa subunit	P26368	22.5	53.8	9.19
4	8.19 ± 2.57	1.68 ± 0.35	4.86	0.0001	eIF3H	O15372	41.2	40.1	6.09
5	5.22 ± 2.84	1.33 ± 0.36	3.91	0.0036	eIF3A	Q14152	15.1	166.9	6.38
6	8.02 ± 1.76	2.28 ± 0.90	3.52	<0.0001	hnRNP Q	O60506	24.4	69.8	8.68
7	2.45 ± 0.29	0.73 ± 0.24	3.35	<0.0001	hnRNP Q	O60506	16.4	69.8	8.68
8	17.11 ± 3.99	5.24 ± 4.26	3.26	<0.0001	hnRNP A2/B1	P22626	36.3	37.5	8.97
9	2.66 ± 1.70	1.00 ± 0.29	2.65	0.0202	eIF3A	Q14152	15.2	166.9	6.38
10	2.37 ± 0.82	0.96 ± 0.19	2.47	0.0010	eIF3F	O00303	28.0	37.7	5.24
11	5.40 ± 1.55	2.27 ± 0.56	2.38	0.0002	hnRNP L	P14866	20.2	64.7	8.46
12	6.77 ± 3.52	2.86 ± 0.68	2.37	0.0113	eIF3G	O75821	16.3	35.9	5.87
13	17.99 ± 9.05	7.89 ± 2.54	2.28	0.0104	eIF3M	Q7L2H7	32.6	42.9	5.41
14	9.28 ± 1.10	4.26 ± 0.71	2.18	<0.0001	Leucine-rich PPR motif-containing protein, mitochondrial	P42704	10.3	159.0	5.81
15	6.78 ± 1.96	3.20 ± 0.75	2.12	0.0005	hnRNP L	P14866	18.3	64.7	8.46
16	2.54 ± 0.55	1.24 ± 0.11	2.05	0.0001	Splicing factor U2AF 65-kDa subunit	P26368	20.0	53.8	9.19
17	17.65 ± 1.23	9.76 ± 1.87	1.81	<0.0001	hnRNP K	P61978	31.3	51.2	5.39
18	32.71 ± 6.34	19.20 ± 6.08	1.70	0.0003	Splicing factor, proline- and glutamine-rich	P23246	19.4	76.2	9.45
19	3.98 ± 0.35	2.34 ± 0.32	1.70	<0.0001	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)	Q99729	17.5	36.3	8.22
20	3.57 ± 0.59	2.13 ± 0.52	1.68	<0.0001	Splicing factor 45	Q96125	17.0	45.2	5.76
21	4.22 ± 1.18	2.52 ± 0.32	1.68	0.0023	hnRNP L	P14866	21.2	64.7	8.46
22	28.44 ± 2.99	17.40 ± 3.82	1.63	<0.0001	hnRNP K	P61978	29.8	51.2	5.39
23	18.62 ± 1.68	11.76 ± 3.01	1.58	<0.0001	ATP-dependent RNA helicase DDX1	Q92499	40.3	83.3	6.81
24	1.81 ± 0.46	1.18 ± 0.35	1.53	0.0052	Fragile X mental retardation syndrome-related protein 1	P51114	15.5	70.0	5.84
25	5.42 ± 0.57	3.60 ± 0.93	1.51	0.0001	Splicing factor U2AF 65-kDa subunit	P26368	26.3	53.8	9.19

[a] Spot numbers correspond to those in Fig. 9.
[b] Intensities of spots are shown as normalized volume ± S.D. (nine gels per group; proanthocyanidin and catechin).
[c] Ratio was calculated using Progenesis Discovery software and expressed as differences of spot intensity in proteins eluted from proanthocyanidin-coupled Sepharose compared with those from catechin-coupled Sepharose.
[d] Statistical difference were determined by Student's *t* test. Values of $p < 0.05$ were considered significant.
[e] Proteins were identified using Mascot with Swiss-Prot database.
[f] References for identified proteins.
[g] Percentage cover of the identified peptide in total tryptic digests.
[h] Theoretical molecular mass from Mascot search results.
[i] Theoretical isoelectric point (pI) from Mascot search results.

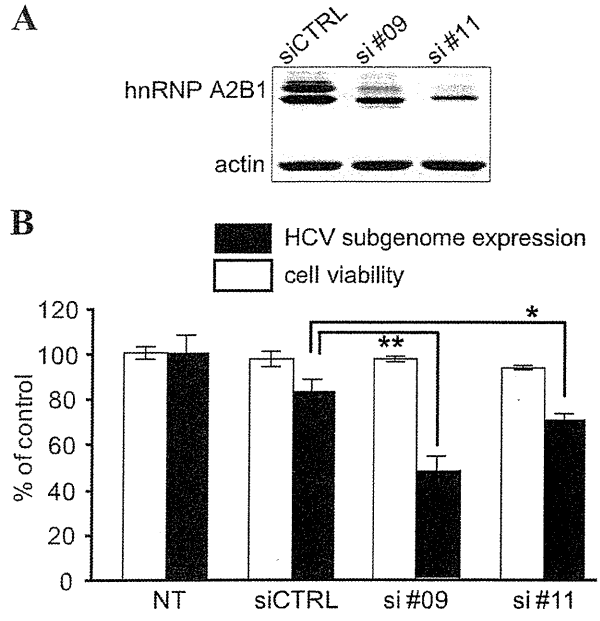

FIGURE 10. **Effects of hnRNP A2/B1 knockdown on HCV subgenome expression in replicon cells.** Results of two siRNA sequences (*si-#09* and *si-#11*) are shown. *A*, effects of siRNA on the expression of hnRNP A2/B1 protein. Same blot was also probed by anti-actin antibody. *B*, effects of siRNA on luciferase activity (HCV subgenome-expression activity) (*closed bars*) and cellular viability (*open bars*). The siRNA concentration is indicated as a logarithmic scale. Values are mean ± S.D. of triplicate experiments. *, $p < 0.05$; **, $p < 0.001$, Student *t* test.

itors of NS3/4A serine protease, and R1479 and HCV-796 are inhibitors of NS5B RNA-dependent RNA polymerase (41, 48–53). NA255 is also an HCV replication inhibitor targeting the host sphingolipid biosynthesis (10). These compounds are relatively low in molecular weight and can be manufactured by organic synthesis. On the other hand, the anti-HCV compound purified from blueberry leaves is a flavan-3-ol polymer with a molecular mass of ~2 kDa. The highly polymerized structure that is required for the efficient inhibition of HCV subgenome expression makes synthesizing the anti-HCV proanthocyanidin difficult. However, because proanthocyanidins are components of many plants and foods, daily intake of proanthocyanidin is possible and may be beneficial against HCV replication in hepatitis C patients. We estimate that fresh blueberry leaf is rich in proanthocyanidin, which accounts for 3–4% of the weight. Moreover, the polymerized status of blueberry leaf-derived proanthocyanidin appears to be suitable for the inhibition of HCV subgenome expression. Therefore, blueberry leaves might have potential as a source of anti-HCV proanthocyanidin.

In summary, we demonstrated that extracts of blueberry leaf possess strong suppressive effects against HCV subgenome expression in a replicon cell system. We identified the inhibitor as a proanthocyanidin oligomer with an mDP value of ~8. Further studies of the mechanism underlying proanthocyanidin-mediated HCV inhibition may open new ways to design novel anti-HCV drugs.

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Research paper

Interferon-γ release assay: A simple method for detection of varicella-zoster virus-specific cell-mediated immunity

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ABSTRACT

Herpes zoster is closely related to decreased varicella-zoster virus (VZV)-specific cell-mediated immunity. We validated a new assay for measuring VZV-specific immunity. We cultured the whole blood of healthy subjects with live attenuated VZV vaccine. Cultured supernatants were harvested at 24-h intervals and assayed for interferon-gamma (IFN-γ) by an enzyme-linked immunosorbent assay (ELISA). The 48-h culture was suitable for estimating IFN-γ release. IFN-γ production was stable after standing for at least 4 h at room temperature. IFN-γ production was observed in whole blood from subjects with recent VZV infection, but not in blood from subjects naïve to the virus. Thus, the IFN-γ release assay may be useful as a new surrogate assay for measuring VZV-specific immunity.

1. Introduction

Varicella-zoster virus (VZV) is a member of the alphaherpesvirus subfamily and is the etiological agent of chicken pox. The virus initially infects the upper respiratory tract or the conjunctiva, then disseminates into the bloodstream through peripheral blood mononuclear cells (PBMCs). The virus is transferred from PBMCs to epithelial cells, resulting in infection of the skin and the characteristic rash of varicella (Arvin et al., 1996). VZV is a neurotropic virus and establishes itself in a dormant form in sensory nerve ganglia. Its reactivation results in herpes zoster (HZ), a localized skin rash following the distribution of the nerves arising from the affected ganglion. The most frequent complication following HZ is chronic and often debilitating pain called postherpetic neuralgia (PHN), which can last for months after the disappearance of a rash. HZ and PHN can have a substantial negative impact on quality of life.

Induction of the innate immune response occurs during acute VZV infection, and cell-mediated and humoral immune responses develop within a few days. VZV-specific antibodies interfere with the initial phases of VZV replication in vivo, as shown by a reduced varicella attack rate (Zaia et al., 1983). Moreover, transplacentally-acquired anti-VZV IgG antibodies are known to protect infants from varicella. In a variety of clinical settings, however, humoral immunity does not appear to correlate with viral reactivation (Arvin and Koropchak, 1980; Webster et al., 1989). Children with agammaglobulinemia have uncomplicated varicella, whereas children with primary cellular immunodeficiency diseases experience progressive varicella infections, which are often fatal (Myers, 1979). A progressive decline in VZV-specific cell-mediated immunity (CMI) that occurs with aging and the absence of an age effect on the levels of VZV antibodies have been demonstrated (Levin et al., 2008). In elderly subjects and immunocompromised patients, the increased risk of HZ is associated with diminished T-cell immunity against VZV (Hata et al., 2002; Levin et al., 2003, 2008; Meyers et al., 1980; Wilson et al., 1992; Zhang et al., 1994). Thus, a substantial amount of evidence indicates that

Abbreviation: CMI, cell-mediated immunity; HZ, herpes zoster; IFN-γ, interferon-gamma; IR, interferon-γ release; PBMCs, peripheral blood mononuclear cells; PHN, postherpetic neuralgia; PHA, phytohemagglutinin; RCF, responder cell frequency; VZV, varicella-zoster virus.

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CMI is more important than humoral immunity in preventing viral infection and reactivation.

VZV is the first human herpes virus for which a vaccine has been developed to prevent primary infection (Takahashi et al., 2008). In addition, it is also considered to be effective in preventing HZ in individuals with suboptimal levels of CMI, such as immunocompromised patients or elderly persons, by boosting CMI (Gershon et al., 1996; Sartori, 2004; Takahashi et al., 2008). Considering the variety of clinical settings in which the VZV vaccine may be administered to prevent HZ, as shown elsewhere (Oxman et al., 2005), the evaluation of VZV-specific CMI is essential for predicting its reactivation.

To date, several methods have been developed to assess VZV-specific immunity, in addition to measuring antibodies to the virus. These methods include the lymphocyte proliferation assay, the responder cell frequency assay, the intracellular cytokine staining assay, the interferon-γ enzyme-linked immunospot assay, the interferon-γ secretion assay, and the skin test. Here, we report a new method, the interferon-γ release (IR) assay, which measures the level of interferon-γ secreted from whole blood after stimulation with the viral particles. This assay is simpler and easier than other in vitro assays, and therefore, may be a candidate for use in point-of-care settings.

2. Materials and methods

2.1. Study subjects

Three healthy adults (age, 28–38 years) and 12 children (age, 9 months to 11 years) were enrolled in the study (Table 1). This study was approved by the ethics committee of Hyogo College of Medicine, and blood collection was performed after obtaining informed consent from all subjects or their legal guardians.

2.2. Antibody titration

Anti-VZV antibody titers in the sera were examined by the indirect immunofluorescence test. VZV (Oka vaccine strain)-infected MRC-5 cells were spotted on glass slides, air dried, and fixed with cold acetone. Serially diluted sera were spotted onto the slides and incubated at 37 °C for 1 h. After the slides were washed with phosphate-buffered saline, fluorescein-conjugated goat antibodies against human IgG or IgM were added to the slides and incubated at 37 °C for 1 h. After the slides were washed, signals were detected by using a fluorescence microscope. The antibody titers were determined as the highest dilution of serum showing a positive signal.

Table 1
IgG antibody titers were estimated by the indirect immunofluorescence test and expressed as the highest dilution of serum showing a positive signal.

Subjects	Age	Antibody titer (IF)
1	32 Y	1:80
2	38 Y	1:40
3	28 Y	1:640
4	1 Y	>1:320
5	4 Y	1:80
6	2 Y	<1:10
7	2 Y	1:40
8	11 Y	1:80
9	3 Y	<1:10
10	11 M	<1:10
11	3 Y	<1:10
12	1 Y	<1:10
13	9 M	<1:10
14	10 M	<1:10
15	1 Y	<1:10

Y, years; M, months.

2.3. IR assay

A live varicella vaccine (Oka strain, BIKEN, lot VZ050) with a titer of more than 1×10^4 plaque forming units per dose was used for the IR assay. Next, 100 μl of heparinized whole blood was plated in flat-bottom microtiter plates and co-cultivated with varying amounts of ultraviolet (UV)-inactivated (2700 J/m^2) varicella vaccine diluted twice with Roswell Park Memorial Institute (RPMI) 1640 medium in a final volume of 200 μl/well. The live vaccine viruses were prepared by reconstitution with distilled water according to the manufacturer's protocol (Biken, Osaka, Japan). Co-cultivations were carried out within 1 h after drawing blood samples from the subjects. One hundred microlitres of culture supernatants was collected 24, 48, or 72 h after cultivation, and IFN-γ concentrations were quantified by using an enzyme-linked immunosorbent assay (ELISA; IFN-γ Assay kit; Biosource International, Camarillo, California) according to the manufacturer's instructions. In order to collect culture supernatants serially, those were harvested from separate parallel cultures prepared for each time points. Either phytohemagglutinin (PHA; final concentration, 2.5 μg/ml) or medium was added to the blood instead of the varicella vaccine as the positive and negative controls, respectively.

3. Results

3.1. VZV-specific antibody titer

We first studied the VZV-specific immune status of all subjects by measuring serum antibody titers. As shown in Table 1, all healthy adults (subjects 1–3) had detectable anti-VZV IgG antibodies. Among the 12 children, including 3 infants, 4 (subjects 4, 5, 7, 8) had the antibodies, while the remaining 8 (subjects 6, 9–15) did not. For 7 of the 8 subjects without antibodies, there was no known history of chickenpox or varicella vaccination. For subject 6, the blood sample was drawn during the acute phase of varicella, and we did not detect anti-VZV IgM antibodies in this subject's blood.

3.2. Evaluation of VZV vaccine amounts in IR assay

We initially attempted the IR assay with a combination of 100 μl of whole blood from 3 subjects (subjects 1–3) and various doses of varicella vaccine in a 24 h culture to assess whether this assay system was feasible. Whole blood produced IFN-γ with increasing amounts of vaccine from 10 to 50 μl, but production of IFN-γ decreased at 100 μl (Fig. 1). These results indicated that the IR assay system using whole blood and the vaccine preparation could be further investigated. Therefore, we subsequently performed the IR assays by culturing 100 μl of whole blood with 50 μl of vaccine and 50 μl of RPMI 1640 medium per well.

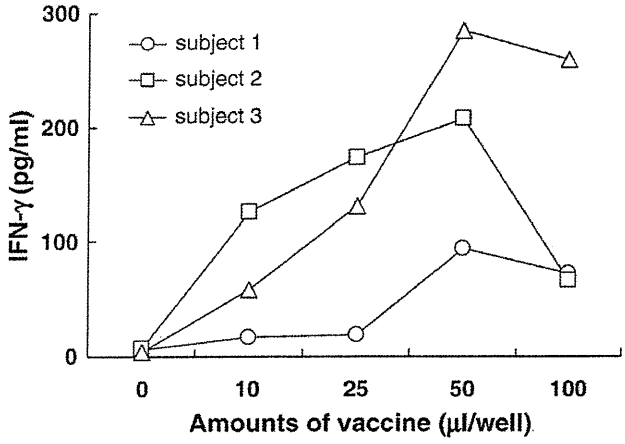

Fig. 1. Evaluation of VZV vaccine amounts suitable for IR assay. Various amounts of vaccine preparation, which were diluted 2 times and UV-irradiated, were mixed with 100 μl of whole blood in a final volume of 200 μl/well. Concentrations of IFN-γ in culture supernatants were measured 24 h later.

3.3. Evaluation of stability of whole blood

In a point-of-care setting, it may not be possible to perform the assay on whole blood samples immediately after collection. In order to investigate the functional stability of whole blood, samples were allowed to stand for 4 or 8 h at room temperature prior to the assay, and the production of IFN-γ in the 24-h cultures were compared with cultures of freshly isolated whole blood samples. After exposure to VZV, samples left standing for 4 h exhibited greater than or equal to 80% of the activity of fresh blood samples. However, blood samples from 2 subjects (1 and 3) lost 80% of their activity after being left standing for 8 h (Fig. 2A). After exposure to PHA, samples from all subjects were found to retain more than 60% activity after 4 h. However, almost all activity in samples from subjects 1 and 3 ceased after 8 h (Fig. 2B). These observations indicated that the assay should be performed on blood samples within 4 h of collection. Thus, all assays were performed within 1 h of obtaining blood samples.

3.4. Evaluation of culture time in IR assay

We next studied IFN-γ release over longer periods, i.e., 48 h, 72 h. As shown in Fig. 3, the amount of IFN-γ produced in the samples from all subjects in response to VZV increased over time, resulting in maximum production in the 72-h culture. However, in the samples of subjects 2 and 3, production almost reached maximum at 48 h. These results showed that whole blood must be cultured with the vaccine for at least 48 h to determine whether IFN-γ is released in response to VZV.

3.5. IR assay of blood from immune and non-immune subjects

Finally, we evaluated IFN-γ release among 12 subjects who had or did not have the anti-VZV antibody. In a 48-h culture of whole blood from 4 subjects who had anti-VZV antibodies (4, 5, 7, and 8) and from subject 6, who did not have antibodies, high amounts of IFN-γ were released. Blood from the remaining 7 subjects who had no antibodies (9–15) produced very low amounts of IFN-γ (Fig. 4). No increase in IFN-γ release was observed in blood from subjects 9–15, even after 72 h in culture

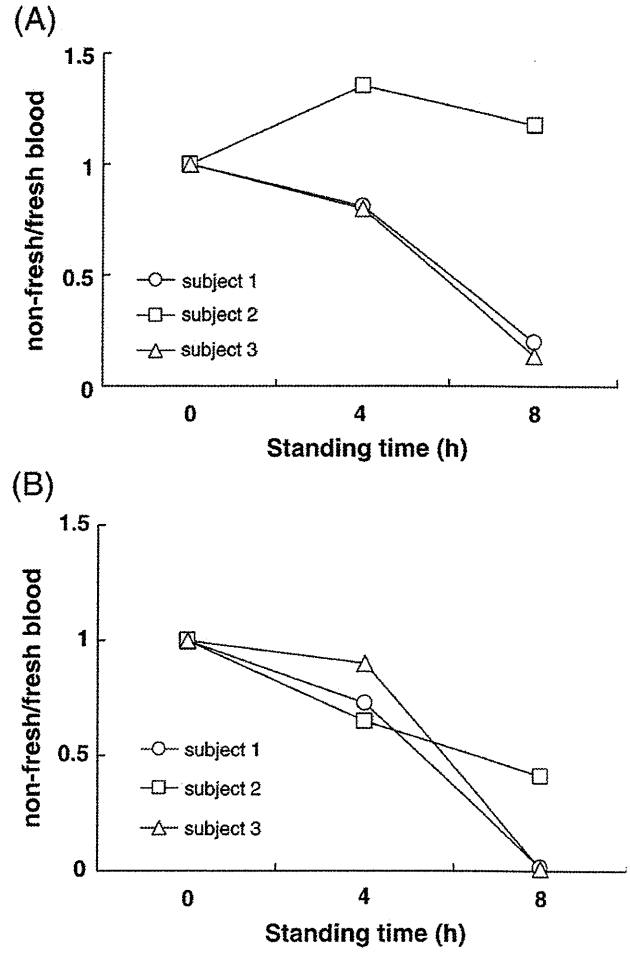

Fig. 2. Functional stability of whole blood samples for IFN-γ production. Freshly isolated whole blood samples from 3 subjects (1, 2, and 3) were kept at room temperature for 4 or 8 h. Whole blood samples (100 μl) were cultured with 100 μl of medium (negative control, NC) or vaccine (VZV), which contained 50 μl of vaccine preparation(A) or phytohemagglutinin (PHA; 5 μg/ml final)(B). Concentrations of IFN-γ in culture supernatants were measured 24 h later. The values were expressed as the ratio of IFN-γ released between freshly isolated blood and non-fresh blood.

(data not shown). When the levels of IFN-γ released and antibody titers of each subject were compared by calculating the correlation coefficient, no relation was observed at all.

4. Discussion

In this study, we assessed a new method that could simply and easily determine VZV-specific immunity. This method is easier to perform than other in vitro assays because the amount of IFN-γ produced in the culture supernatant can be directly determined following the incubation of the patient's whole blood with the commercially available VZV vaccine. Most importantly, we could clearly differentiate VZV-immune subjects from non-immune subjects by the level of IFN-γ production in their blood. Although further studies using a larger number of blood samples are needed, the results in Fig. 3 show that an IFN-γ level of over 100 pg/ml appears to indicate immunity against VZV. Because of its ability to induce Th1-dominant immunity, IFN-γ is considered to be a marker of CMI. Thus, the level of IFN-γ in the blood reflects the degree of

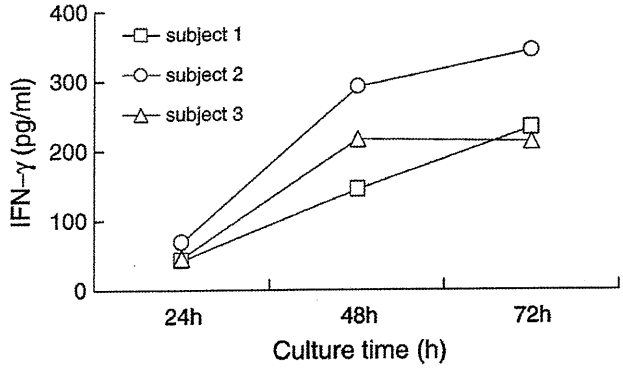

Fig. 3. IFN-γ production from the whole blood of 3 subjects after various culture times. IR assays were performed as described in the legend of Fig. 2, but culture supernatants were harvested after 48 h and 72 h of culture, in addition to the 24-h culture.

induction of CMI by the corresponding antigens. This principle was recently applied to confirm tuberculosis infection (Dinnes et al., 2007). Therefore, we concluded that subjects showing significant IFN-γ release in their blood (subjects 4–8) have CMI against VZV, but others (subjects 9–15) do not. Thus, the IR assay may be a candidate for measuring VZV-specific CMI in point-of-care settings.

Of particular interest are the observations for subject 6. The blood sample of this subject was drawn during the acute phase of the disease, and no antibodies against VZV, including IgM, were detected; however, a high level of IFN-γ release was observed. These findings indicate that the IR assay can detect early CMI response prior to antibody response, further indicating the utility of this method as a tool for early diagnosis.

The goal of measuring VZV-specific CMI is to predict the threshold level of immunity at which the reactivation of VZV cannot be prevented, thus causing HZ. However, to date the exact threshold value has not been determined. Levin et al. (2008) recently demonstrated an inverse correlation between the level of VZV-specific CMI and the likelihood of developing HZ by using the responder cell frequency (RCF) and ELISPOT assays. However, they did not identify a particular level of VZV-specific CMI, because of the small number of HZ cases in the study and an inability to collect samples during the onset of HZ. On the other hand, Hata et al. (2002) used the LP assay and reported intriguing observations in the analyses of patients who underwent hematopoietic-cell transplantation. They showed that the risk of HZ decreased for each unit increase above the stimulation index of 1.6; a stimulation index above 5.0 correlated with greater than 93% protection. As shown by Hata et al. (2002), monitoring VZV-specific CMI in patients with hematopoietic-cell transplantation would be a good experimental system to determine the threshold level of immunity.

In conclusion, we have developed a new assay that measures IFN-γ release induced by VZV antigens, that reflects VZV-specific CMI, and that correlates well to both history of varicella and the VZV-immune status of subjects. Therefore, the IR assay may be a good alternative for determining VZV-specific CMI.

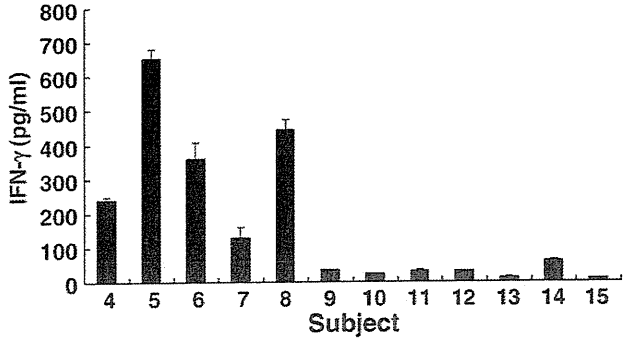

Fig. 4. IFN-γ production from the whole blood of 12 subjects. IR assays were performed as described in the legend of Fig. 2, but culture supernatants were harvested after 48 h.

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