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

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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-electrophoresis showed 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 subgenomic 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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² 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; APCL, atmospheric pressure chemical ionization; mDP, mean degree of polymerization; IC50, concentration required for 50% inhibition; CC50, concentration required for 50% cytotoxicity; elf3, eukaryotic translation initiation factor 3; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; IRES, internal ribosome entry site; DIGE, differential gel electrophoresis.
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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% CO₂. 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 (IC₅₀). Specific activity was calculated as a reciprocal number of IC₅₀ (1/IC₅₀). 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 (CC₅₀).

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 (acetoni-trite) 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 N₂ gas, 1.5 or 2.0 liters/min; drying N₂ 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 NH₄Fe(SO₄)₂·12H₂O dissolved in 2 ml H₂O. 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 N₂ 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).

Thiolyis Analysis—Thiolyis 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 epicatechin. 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 elF3H polyclonal antibodies (Novus Biologicals, Littleton, CO), and

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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) aceton (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.
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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 × 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% bromophenol 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 4–7) and 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, hnRNQ, elf3F, elf3G, elf3H, 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′-CGGUGGAAAUUCGGAACCA-3′, Target #11: 5′-GGAGGUAGUGGCGCAA-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 IC50 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 IC50 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
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(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. 2A). To purify the most active component, we initially separated the CMW-W isocratic at 30% acetonitrile (17 min) with some minor inhibitory activities eluted around 90% of acetonitrile (a 15–100% gradient of acetonitrile) was used. The data indicated that a strong inhibitory activity 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 IC₅₀ 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 CC₅₀ value of the cytotoxicity of LC3 was 18.5 μg/ml, and the selective index, which was calculated by dividing CC₅₀ by IC₅₀, 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 C₃₉H₂₄O₁₂ (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 anthocyaninidin 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. 5A). Indeed, MS/MS spectra of this peak were identical to those of the cyanidin standard (Fig. 5B). 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. 5A, 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]⁻ 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 IC₅₀ 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 CC₅₀ value of the cytotoxicity of LC3 was 18.5 μg/ml, and the selective index, which was calculated by dividing CC₅₀ by IC₅₀, was 212.6, showing a 16.5-fold higher selective index value compared with initial methanol extracts (Fig. 3).

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### TABLE 1

Purification of HCV subgenome expression inhibitory activity in blueberry leaf

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<td>LC 3rd</td>
<td>2.9</td>
<td>0.087</td>
<td>11.49</td>
<td>62.87</td>
<td>33.33</td>
</tr>
</tbody>
</table>
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 proanthocyanidins 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 (supplementary 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).

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. 9A and Table 4). Although other possible candidate spots were also suggested in a rectangular portion (Fig. 9A), 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
Blueberry Leaf Proanthocyanidin Suppresses HCV

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DP or mDP</th>
<th>Subgenome expression, IC50</th>
<th>Cytotoxicity, CC50</th>
<th>Ratio, CC50/IC50</th>
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</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>1</td>
<td>16.18</td>
<td>100.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1</td>
<td>27.32</td>
<td>113.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Epigallocatechin-gallate</td>
<td>1</td>
<td>14.61</td>
<td>41.68</td>
<td>2.9</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>2</td>
<td>&gt;25.0</td>
<td>&gt;25.0</td>
<td>–</td>
</tr>
<tr>
<td>Purified proanthocyanidin from blueberry leaf (LC3 fraction)</td>
<td>7.7</td>
<td>0.087</td>
<td>18.5</td>
<td>212.0</td>
</tr>
</tbody>
</table>

a Epicatechin dimer.

Blueberry Leaf Proanthocyanidin Suppresses HCV

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 SCH50304 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 pl (rectangular regions) were not sufficiently separated and were not analyzed.

### Table 4: Proteins with higher affinity to blueberry proanthocyanidin than to catechin

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Intensity a (×10^3)</th>
<th>Ratio b</th>
<th>p-value ^c</th>
<th>Protein name d</th>
<th>Accession number e</th>
<th>Coverage f</th>
<th>Molecular mass g (kDa)</th>
<th>pl h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.69 ± 2.98</td>
<td>0.99 ± 0.24</td>
<td>5.73</td>
<td>0.0015</td>
<td>Leucine-rich PPR motif-containing protein, mitochondrial</td>
<td>Q14152</td>
<td>16.9</td>
<td>6.38</td>
</tr>
<tr>
<td>2</td>
<td>6.96 ± 1.33</td>
<td>1.39 ± 0.30</td>
<td>5.00</td>
<td>&lt;0.0001</td>
<td>hnRNPL Q</td>
<td>O60506</td>
<td>69.8</td>
<td>8.68</td>
</tr>
<tr>
<td>3</td>
<td>5.63 ± 0.71</td>
<td>1.15 ± 0.36</td>
<td>4.88</td>
<td>&lt;0.0001</td>
<td>Splicing factor U2AF 65-kDa subunit</td>
<td>P26368</td>
<td>53.8</td>
<td>9.19</td>
</tr>
<tr>
<td>4</td>
<td>8.19 ± 2.57</td>
<td>1.68 ± 0.35</td>
<td>4.86</td>
<td>&lt;0.0001</td>
<td>eIF3H</td>
<td>O15372</td>
<td>41.0</td>
<td>6.09</td>
</tr>
<tr>
<td>5</td>
<td>5.22 ± 2.84</td>
<td>1.33 ± 0.36</td>
<td>3.91</td>
<td>&lt;0.0006</td>
<td>eIF3A</td>
<td>Q14152</td>
<td>16.9</td>
<td>6.38</td>
</tr>
<tr>
<td>6</td>
<td>5.02 ± 1.76</td>
<td>2.28 ± 0.90</td>
<td>3.52</td>
<td>&lt;0.0001</td>
<td>hnRNPL Q</td>
<td>O60506</td>
<td>69.8</td>
<td>8.68</td>
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<tr>
<td>7</td>
<td>2.45 ± 0.29</td>
<td>0.73 ± 0.24</td>
<td>3.35</td>
<td>&lt;0.0001</td>
<td>hnRNPL Q</td>
<td>O60506</td>
<td>69.8</td>
<td>8.68</td>
</tr>
<tr>
<td>8</td>
<td>17.11 ± 3.99</td>
<td>5.24 ± 4.26</td>
<td>3.26</td>
<td>&lt;0.0001</td>
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<td>37.5</td>
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<tr>
<td>9</td>
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<td>eIF3A</td>
<td>Q14152</td>
<td>16.9</td>
<td>6.38</td>
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<tr>
<td>10</td>
<td>2.37 ± 0.82</td>
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<td>11</td>
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<td>2.27 ± 0.56</td>
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<td>&lt;0.0002</td>
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<td>eIF3M</td>
<td>Q71247</td>
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<td>14</td>
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<tr>
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<td>2.12</td>
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<td>16</td>
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<td>1.24 ± 0.11</td>
<td>2.05</td>
<td>&lt;0.0001</td>
<td>Splicing factor U2AF 65-kDa subunit</td>
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<tr>
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<tr>
<td>18</td>
<td>32.71 ± 6.34</td>
<td>19.20 ± 6.08</td>
<td>1.70</td>
<td>0.0003</td>
<td>Splicing factor, proline- and glutamine-rich</td>
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<tr>
<td>19</td>
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<td>2.34 ± 0.32</td>
<td>1.70</td>
<td>&lt;0.0001</td>
<td>Heterogeneous nuclear ribonucleoprotein A/B (hnRNPL A/B)</td>
<td>Q99729</td>
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<tr>
<td>20</td>
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<td>2.13 ± 0.52</td>
<td>1.68</td>
<td>&lt;0.0001</td>
<td>Splicing factor 4F</td>
<td>Q96425</td>
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<td>21</td>
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<td>1.63</td>
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<td>P61978</td>
<td>51.2</td>
<td>5.39</td>
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<td>23</td>
<td>18.62 ± 1.68</td>
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<td>ATP-dependent RNA helicase DDX1</td>
<td>Q93299</td>
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<tr>
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<td>0.0052</td>
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<td>Splicing factor U2AF 65-kDa subunit</td>
<td>P26368</td>
<td>53.8</td>
<td>9.19</td>
</tr>
</tbody>
</table>

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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 Theoretical molecular mass from Mascot search results.

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1 Statistical difference were determined by Student’s t test. Values of p < 0.05 were considered significant.

2 Proteins were identified using Mascot with Swiss-Prot database.

3 References for identified proteins.

4 Percentage cover of the identified peptide in total tryptic digests.

5 Theoretical molecular mass from Mascot search results.

6 Theoretical isoelectric point (pl) from Mascot search results.
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A

hnRNP A2/B1

actin

siCTRL

si #09

si #11

HCV subgenome expression

cell viability

% of control

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.

REFERENCES


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