SuperSignal West Dura Extended Duration Substrate (Pierce, 34075) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077) according to standard protocols.

Preparation of HCV pseudoparticles (HCVpp) HCVpp were produced as previously described (33). Briefly, 293T cells were cotransfected with a Gag-Pol packaging vector (Gag-Pol 5349), reporter vector (Luc 126), and glycoprotein (HCV E1 and E2)-expressing vector [genotype 2a, the JFH1 strain; genotype 1b, the TH strain (34)]. The medium was collected from the transfected cell cultures and used as the HCVpp source.

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Results

One-shot treatment with caffeic acid to the HCV-infected cells inhibits the propagation of HCV.

To investigate whether the coffee extract inhibited HCV propagation, we examined the amount of HCV particles released into the medium after the treatment of HCV-infected cells (Figure 1A). Huh7.5.1-8 cells were infected with HCV particles for 3 h at an moi of 0.1. After washing to remove free HCV particles, the HCV-infected

cells were incubated in the normal culture medium containing 1% coffee extract at 37°C for 1 h. Thereafter, the cells were cultured in the normal culture medium without the coffee extract for 3 and 4 days. HCV particles released into the medium were investigated at 3 and 4 days post-infection (dpi) (Figure 1A). There were minimal abnormalities in cell morphology and viability under these conditions. The 1-h coffee extract treatment considerably decreased the amount of HCV particles released into the medium ($p<0.004$ at 3 dpi and $p<0.003$ at 4 dpi) (Figures 1B & C).

Furthermore, we investigated the effect of coffee extract treatment on the intracellular levels of HCV core and NS3 proteins in the infected cells 4 dpi (Figure 1D). When cells were treated with 1% coffee extract for 1 h after HCV infection, levels of HCV core and NS3 proteins were found to be low, whereas these were more abundant in the HCV-infected cells without the treatment. The levels of total proteins in the coffee extract-treated cells was similar to that of the untreated cells (Figure 1E), suggesting that coffee extract had little effect on the cell viability and proliferation. These results indicated that HCV propagation was markedly inhibited by the 1-h coffee extract treatment.

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Chlorogenic acid is a major component of coffee (approximately 5%–10%), and it is metabolically decomposed to caffeic acid and D(−)-quinic acid after absorption. As caffeic acid is present in human plasma after the consumption of coffee (30), we focused our study on this compound. And to clarify the effect of caffeic acid on HCV propagation, we investigated the abundance of HCV particles released from infected cells at 3 and 4 dpi, following a 1-h treatment with 0.1% caffeic acid and found that the number of HCV particles released was substantially decreased at 3 and 4 dpi ($p<0.006$ at 3 dpi and $p<0.006$ at 4 dpi), which was similar to the results observed after treatment with the coffee extract (Figure 1B & C). The intracellular levels of HCV core and NS3 proteins in the HCV-infected cells at 4 dpi were also a markedly decreased by the caffeic acid treatment (Figure 1D). Caffeic acid took little effect on the level of total proteins of the cells (Figure 1E), suggesting that caffeic acid had little effect on the cell viability and proliferation under these conditions. There were minimal abnormalities in cell morphology and viability under these conditions. These results indicate that, in addition to the 1% coffee extract, the one-shot 0.1% caffeic acid treatment also inhibited HCV propagation.

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HCV propagation is inhibited by continuous treatment with a lower caffeic acid concentration

Next, we investigated whether HCV particle release was decreased by a continuous treatment with lower concentrations of coffee extract and caffeic acid compared with the initial concentrations (Figure 2). Huh7.5.1-8 cells were infected with naïve HCV particles for 3 h (moi = 0.1). After infection, cells were cultured in the presence of 0.01% coffee extract or 0.001% caffeic acid for 3 and 4 days (Figure 2A), after which the level of HCV particles released into the medium was estimated at both time points. As before, minimal abnormalities in cell morphology and viability under these conditions were observed. At 4 dpi, the abundance of HCV particles released into the medium was drastically decreased in the presence of both 0.01% of coffee extract and 0.001% of caffeic acid ($p<0.006$ and $p<0.005$, respectively) (Figure 2B), with a corresponding decrease in the intracellular level of HCV core and NS3 proteins (Figure 2C). Little difference of total proteins in the cell lysate was observed between the compound-treated and the untreated HCV-infected cells (Figure 2D), suggesting the

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little effect of these compounds on the cell viability and proliferation. These results were similar to those of the one-shot treatment experiments, as they indicate that continuous caffeic acid treatment also inhibited HCV propagation.

Caffeic acid inhibits the initial stage of HCV infection

The treatment of HCV-infected cells with caffeic acid resulted in a decreased release of HCV particles. This inhibitory effect was observed even with the one-shot 1-h caffeic acid treatment. Considering these results, it is possible that caffeic acid can inhibit the initial stage of HCV infection (from viral entry to viral genome translation). To investigate this possibility, we employed infectious HCVpp (35). The entry of HCV into target cells depends on the envelope glycoproteins. HCVpp is assembled by displaying unmodified and functional HCV glycoproteins on retroviral core particles to mimic the initial stage of HCV infection. Because a firefly luciferase gene was introduced into the HCVpp viral genome, HCVpp entry into target cells can be estimated by firefly luciferase measurement. Therefore, cells will express active luciferase only when it is translated from the viral genome following HCVpp infection

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of target cells.

HCVpp-infected cells were washed to remove free HCVpp, followed by a 1-h incubation in medium containing either 1% coffee extract or 0.1% caffeic acid. Cells were washed again and then cultured in the normal culture medium for 2 days, at which point the cellular luciferase activity was measured (Figure 3A). When the HCVpp-infected cells were treated with 1% coffee extract or 0.1% caffeic acid for 1 h, the luciferase activity was markedly decreased compared with the untreated HCVpp-infected cells ($p<0.002$ and $p<0.001$, respectively). These results suggest that coffee extract and caffeic acid severely inhibit the initial stage of HCV infection.

We then performed the similar experiment to investigate the effect of continuous caffeic acid treatment at a lower concentration on the initial stage of HCV infection (Figure 3B). Huh7.5.1-8 cells were infected with HCVpp for 3 h. After washing the cells to remove free HCVpp, they were cultured in the presence of 0.001% caffeic acid for 2 days. Thereafter the activity of cellular luciferase was measured. When the HCVpp-infected cells were treated with caffeic acid, the activity of cellular luciferase decreased ($p<0.005$) (Figure 3B), thus indicating that continuous caffeic acid

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treatment also inhibits the initial stage of HCV infection.

Next, we focused on another of the major HCV genotypes, particularly, 1b (Figure 3C). We prepared HCVpp genotype 1b and infected Huh7.5.1-8 cells for 3 h as described above. After infection and removal of free HCVpp, the cells were treated for 1 h with either 1% coffee extract or 0.1% caffeic acid. Following treatment, the cells were washed and further cultured in normal medium for 2 days, at which point the cellular luciferase activity was measured. As was the case for HCVpp genotype 2a, the HCVpp genotype 1b-infected cells treated with 1% coffee extract or 0.1% caffeic acid had drastically decreased cellular luciferase activity ($p<0.001$ and $p<0.001$, respectively). These results suggested that the inhibitory effect of coffee extract and caffeic acid on the initial stage of HCV infection is independent of viral genotype.

Higher concentration of *p*-coumaric acid inhibits the propagation of HCV

We further investigated the anti-HCV effects of additional organic acids from the coffee extract, including *p*-coumaric acid, quinic acid, and nicotinic acid (Figure 4). Huh7.5.1-8 cells were infected with HCV particles for 3 h and cultured in the presence

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of 0.1% *p*-coumaric acid, D(−)-quinic acid, or nicotinic acid for 3 and 4 days. The number of HCV particles released into the medium was then examined. Only *p*-coumaric acid strongly inhibited the HCV particle release ($p<0.008$ at 3 dpi and $p<0.001$ at 4 dpi), although these organic acids were used at a concentration 100-fold higher than that of caffeic acid. Little difference of total proteins in the cell lysate was observed among the compound-treated and the untreated HCV-infected cells (Figure 4), suggesting little effect of these compounds on the cell viability and proliferation.

Discussion

In this study, we used an *in vitro* naïve HCV particle-infection and production system and demonstrated that coffee extract and caffeic acid inhibited the propagation of HCV. The one-shot treatment of HCV-infected cells with 1% coffee extract and 0.1% caffeic acid for 1 h inhibited HCV propagation at 3 and 4 dpi. Moreover, continuous treatment of HCV-infected cells with 0.01% coffee extract and 0.001% caffeic acid demonstrated the similar inhibition. We further demonstrated that caffeic acid inhibited the initial stage of HCV infection. Our results strongly suggest that caffeic acid derived

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from the coffee extract inhibits HCV propagation.

The one-shot treatment with 0.1% caffeic acid for 1 h effectively inhibited HCV propagation *in vitro*. Interestingly, the continuous treatment with 0.001% caffeic acid had a similar effect. Coffee consumption results in increased caffeic acid in human total plasma (30, 36). The caffeic acid concentration in human total plasma at approximately 1-h after coffee consumption is approximately 80–116 nM, which gradually decreases (approximately 50 nM at 8 h) and reduces to almost zero at 12 h. The caffeic acid concentration used for the continuous treatment (0.001%) was approximately 55 nM. Thus, the increased caffeic acid concentration in human plasma after coffee intake may inhibit HCV infection and/or propagation in humans. According to human studies, the amount of coffee consumed per day correlates with a decreased risk of chronic liver diseases (11, 15, 28). Furthermore, coffee consumption is associated with a response to peginterferon and ribavirin therapy in patients with chronic hepatitis C (27). The results presented in this study suggest that the plasma concentration of caffeic acid is important for the inhibition of HCV infection and HCV propagation.

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We found that the one-shot treatment with the coffee extract and caffeic acid severely inhibited the initial stage of HCV infection, an effect that is independent of viral genotype. Therefore, this may partly contribute to the decrease in HCV propagation. Considering that continuous treatment with lower concentrations of both substances also inhibited HCV propagation, they may affect intracellular processes essential for HCV propagation, such as polyprotein processing; RNA replication; and virion assembly, transport, or release, in addition to viral entry and viral polyprotein translation. It is possible that caffeic acid inhibits in part RNA-replication in HCV-infected cells, since it has been reported that caffeic acid inhibits HCV-replication in HCV subgenomic replicon cells at higher concentration (EC50, about 37 μM) (37). Further studies are warranted to clarify the step(s) that is inhibited by these treatments.

The 1-h treatment with coffee extract or caffeic acid following HCV infection was sufficient to severely inhibit HCV propagation. HCV cellular entry is mediated via many receptors, including (at least) human CD81, low-density lipoprotein receptor, occludin, scavenger receptor class B type I, claudin-1, and the Niemann–Pick C1-like 1 cholesterol absorption receptor. The time course of HCV entry and the mechanism by

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which these receptors contribute to HCV entry remain unclear. Further studies are required to clarify these questions.

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Conflict of interest: None to declare.

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Figure Legends.

Figure 1. HCV propagation is inhibited by one-shot treatment of HCV-infected cells with coffee extract and caffeic acid for 1 h. (A) The schedule for exposing Huh7.5.1-8 cells to naïve HCV particles and compounds is schematically represented. Huh7.5.1-8 cells were infected with naïve HCV particles (JFH-1, genotype 2a) for 3 h. After washing free HCV particles, HCV-infected cells were incubated in the culture medium containing 1% coffee extract or 0.1% caffeic acid at 37°C for 1 h. The medium containing HCV-infected cells incubated in the culture medium for 1 h was used as the positive control. After washing the cells, they were incubated at 37°C in the culture

medium for the indicated time points. Black and dotted bars indicate the intervals with and without treatments, respectively. (**B**) The effect of 1% coffee extract and 0.1% caffeic acid on HCV particle released into the medium. After HCV infection, cells were treated with 1% coffee extract (open squares) or 0.1% caffeic acid (open triangles) at 37°C for 1 h. The medium containing cells incubated the culture medium for 1 h was used as the positive control (closed squares). The relative amounts of HCV core proteins with that of HCV particles released from nontreated HCV-infected cells at 3 dpi set to 100% is shown. Error bars indicated the standard error of the mean (S.E.M., n = 4). (**C**) The relative amount of HCV core proteins in the medium at 3 dpi. (**D**) The intracellular level of HCV-related proteins in HCV-infected cells under the same conditions at 4 dpi. HCV core and NS3 proteins in the cell lysates were visualized by immunoblotting using the appropriate antibodies. GAPDH was employed as the loading control. (E) Little effect of 1% coffee extract and 0.1% caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicated the standard error of the mean (S.E.M., n = 4).

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Figure 2. HCV propagation is inhibited by continuous treatment of HCV-infected cells with coffee extract and caffeic acid. (**A**) The schedule for exposing Huh7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. Huh7.5.1-8 cells were infected with naïve HCV particles (JFH-1, genotype 2a) for 3 h. After washing free HCV particles from the medium, HCV-infected cells were cultured in medium containing 0.01% coffee extract or 0.001% caffeic acid at 37°C. Black and dotted bars indicate the intervals with and without treatments, respectively. (**B**) The amounts of HCV particles released into the medium in the presence of 0.01% coffee extract and 0.001% caffeic acid is shown. After HCV infection, cells were cultured in the normal culture medium in the presence of 0.01% coffee extract (open squares) or 0.001% caffeic acid (open triangles) at 37°C. The medium containing HCV-infected cells incubated the culture medium for 1 h was used as the positive control (control, closed squares). The relative amount of HCV core proteins with that of HCV particles released from nontreated HCV-infected cells at 3 dpi set as 100% is shown. Error bars indicated the standard error of the mean (S.E.M., n = 4). (**C**) The intracellular levels of

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HCV-related proteins in infected cells under the same conditions at 4 dpi. HCV core and NS3 proteins were visualized by immunoblotting with the appropriate antibodies. GAPDH was employed as the loading control. (D) Little effect of continuous treatment of HCV-infected cells with coffee extract and caffeic acid on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). Error bars indicated the standard error of the mean (S.E.M., n = 4).

Figure 3. Caffeic acid inhibits the initial stage of HCV infection. (A) The one-shot treatment of caffeic acid inhibits the initial stage of HCV infection. The schedule for exposing Huh7.5.1-8 cells to compounds and HCV pseudoparticles (HCVpp) (genotype 2a) was the same as that shown in Figure 1A. The medium containing HCVpp-infected cells incubated in the normal culture medium without these compounds was used as the positive control. Relative activities of luciferase with the activity of the nontreated HCVpp-infected cells (control) was set to 100% are shown. The data from three independent experiments are shown. Error bars indicated the standard error of the mean

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(S.E.M.). (**B**) Continuous caffeic acid treatment also inhibits the initial stage of HCV infection. The schedule for exposing Huh7.5.1-8 cells to compounds and HCVpp (genotype 2a) is the same as that shown in Figure 2A. The medium containing HCVpp-infected cells incubated in the normal culture medium was used as the positive control. Relative activities of luciferase with the activity of the nontreated HCVpp-infected cells (control) set to 100% are shown. (**C**) Caffeic acid inhibits the initial stage of HCVpp genotype 1b infection. The experiments were performed as described in (**A**) using HCVpp genotype 1b instead of HCVpp genotype 2a.

Figure 4. High concentration of *p*-coumaric acid inhibits the HCV propagation. (**A**) The schedule for exposing Huh7.5.1-8 cells to compounds and naïve HCV particles is schematically represented. (**B**) The effects of D-(−)-quinic acid, nicotinic acid, and *p*-coumaric acid on the HCV propagation were investigated. The relative amount of the HCV core protein in the medium with its amount in the medium without compounds at 3 dpi set to 100% is shown. The data from three independent experiments are shown. Error bars indicated the standard error of the mean (S.E.M.). (C) Little effect of these

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compounds on total proteins in HCV-infected cells. The schedule for exposing Huh7.5.1-8 cells to HCV particles and compounds was the same as that shown in (A). The data from two independent experiments are shown. Error bars indicated the standard error of the mean (S.E.M., n = 4).

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