The Green Tea Polyphenol, Epigallocatechin-3-Gallate, Inhibits Hepatitis C Virus Entry

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Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma. Current antiviral therapy fails to clear infection in a substantial proportion of cases. Drug development is focused on nonstructural proteins required for RNA replication. Individuals undergoing orthotopic liver transplantation face rapid, universal reinfection of the graft. Therefore, antiviral strategies targeting the early stages of infection are urgently needed for the prevention of HCV infection. In this study, we identified the polyphenol, epigallocatechin-3-gallate (EGCG), as an inhibitor of HCV entry. Green tea catechins, such as EGCG and its derivatives, epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), have been previously found to exert antiviral and antioncogenic properties. EGCG had no effect on HCV RNA replication, assembly, or release of progeny virions. However, it potently inhibited Cell-culture–derived HCV (HCVcc) entry into hepatoma cell lines as well as primary human hepatocytes. The effect was independent of the HCV genotype, and both infection of cells by extracellular virions and cell-to-cell spread were blocked. Pretreatment of cells with EGCG before HCV inoculation did not reduce HCV infection, whereas the application of EGCG during inoculation strongly inhibited HCV infectivity. Moreover, treatment with EGCG directly during inoculation strongly inhibited HCV infectivity. Expression levels of all known HCV (co-)receptors were unaltered by EGCG. Finally, we showed that EGCG inhibits viral attachment to the cell, thus disrupting the initial step of HCV cell entry. *Conclusion:* The green tea molecule, EGCG, potently inhibits HCV entry and could be part of an antiviral strategy aimed at the prevention of HCV reinfection after liver transplantation. (HEPATOLOGY 2011;54:1947-1955)

Infection with the hepatitis C virus (HCV) is a major cause of chronic liver disease and the major indication of liver transplantations worldwide. Approximately 160 million people are chronically infected with HCV, representing approximately 2% of the world population, whereas in some countries up to 15%-20% of the population is infected.[1] Chronic HCV infection causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Standard therapy currently consisting of a combination of pegylated interferon-alpha (IFN-α) with ribavirin will soon also include an HCV nonstructural protein (NS)3/4A protease inhibitor.[2] This addition is highly expected to improve response rates, especially in HCV genotype 1–infected individuals. However, therapy will most likely still remain expensive, fraught with side effects, and may still fail to clear infection in a substantial proportion of cases. Individuals undergoing orthotopic

Abbreviations: INN, boceprevir; CD, cluster of differentiation; CLDN1, claudin-1; cpm, counts per minute; CyA, cyclosporine A; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; EGF-R, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; FBS, fetal bovine saerum; ffu, focus-forming units; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, cell-culture–derived HCV; HCVpp, HCV pseudoparticles; HPM, hepatocyte plating media; IC_{50}, half-maximal inhibitory concentration; IFN-α, interferon-alpha; JFH1, Japanese fulminant hepatitis 1; MLV, murine leukemia virus; MOI, multiplicity of infection; NS, nonstructural protein; NTRs, nontranslated regions; OCLN, occluding; OLT, orthotopic liver transplantation; oxLDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; PHHs, primary human hepatocytes; RFP, red fluorescent protein; SR-BI, scavenger receptor class B type 1; VSV-G, vesicular stomatitis virus glycoprotein.

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liver transplantation (OLT) for complications of HCV infection pose a particular clinical problem: Graft reinfection with HCV occurs in nearly all cases, and long-term outcomes are unsatisfactory.[3] The pharmacological repression of immune function after OLT results in enhanced viral replication. Moreover, some immunosuppressants have additional nonimmune-mediated proviral effects, as we have demonstrated for glucocorticoids.[4] Prevention of graft reinfection, as routinely achieved in the case of hepatitis B, is a major clinical goal, but will likely require efficient pharmacological means of preventing viral entry into hepatocytes. Such compounds are, so far, not available.

HCV is a highly variable enveloped RNA virus of the *Flaviviridae* family that infects hepatocytes and establishes a chronic infection in the majority of cases. The HCV genome, 9.6 kilobases in size, encodes for a single polyprotein cleaved by cellular and viral proteases into 10 different proteins: core, E1, E2 (structural proteins), p7, and the nonstructural proteins (NSs), NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The single open reading frame (3,000 amino acids) encoded by the HCV genome is flanked by nontranslated regions (NTRs) at the 5′- and 3′-end.

Cell-culture–derived HCV particles (HCVcc), based on the Japanese fulminant hepatitis 1 (JFH1) strain of HCV genotype 2a, are infectious both *in vitro* and *in vivo* and, therefore, are a widely used model for the complete replication cycle of HCV.[5-7] Unlike other HCV isolates, JFH1 replicates very efficiently in Huh-7.5 cells in the absence of cell-culture–adaptive mutations. Construction of chimeric virus genomes encoding JFH1-derived nonstructural proteins and structural protein to form the J6/CF strain (genotype 2a) has further improved the efficiency of the HCV infection model.[8] This system allows the assessment of the impact of antiviral agents on HCV RNA replication, virus production, and infectivity.

Green tea catechins, such as epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), have been found to have antiviral and antioncogenic properties.[9] Functional and structural differences are attributed to the number of hydroxyl groups on the B-ring and the presence or absence of a galloyl moiety. For EGCG, a major component of green tea, inhibition of herpes simplex virus type 1 and 2, enterovirus 71, human immunodeficiency virus, influenza A, and other viruses has been reported.[10-14] The mechanism of the observed broad antiviral effect is not known, but has been suggested to differ between viruses. This study was conducted to evaluate the effects of green tea catechins on HCV and to explore the mechanism underlying their antiviral effects.

Our results reveal that EGCG, but not other green tea catechins, is an inhibitor of HCVcc that does not interfere with genome replication. Instead, EGCG specifically targets viral cell entry (i.e., the NS3/4A-independent initial stage of the viral replication cycle) into both hepatoma cell lines and primary human hepatocytes. Furthermore, we showed that EGCG inhibits viral attachment to the target cell as well as cell-to-cell transmission between adjacent cells.

Materials and Methods

Compounds. EGCG, ECG, EC, and EGC were purchased from Sigma-Aldrich (Seelze, Germany). Cyclosprine A (CyA) was provided by Novartis (Basel, Switzerland), and boceprevir (INN) was provided by the Institute Pasteur Korea (Seongnam, Korea).

Plasmids and Viruses. Plasmids pFK-Jc1, pFK-JFH1, and H77/JFH1 have been described recently.[8,15] The construct, Luc-Jc1, is a bicistronic firefly luciferase reporter virus that encodes a chimeric HCV polyprotein consisting of codons 1-846 derived from J6/CF combined with codons 847-3033 of JFH1.[16]

Cell Culture. Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS), 1× nonessential amino acids (Invitrogen), 100 μg/mL of streptomycin (Invitrogen), and 100 IU/mL of penicillin (DMEMcomplete; Invitrogen).

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Additional Supporting Information may be found in the online version of this article.

Primary human hepatocytes were purchased from Invitrogen and resuspended in hepatocyte plating media (HPM; 500 mL of DMEM high glucose and 10% FBS) and plated at a concentration of at 2.5×10^5 cells/cm^2. We used diluted collagen (type 1, rat tail; BD, Heidelberg, Germany) (50 μg/mL in 0.02 N of acetic acid) for coating coverslips and plates in approximately 10 mL at room temperature for 1 hour. The collagen solution was then removed, and the plastic surface was rinsed twice with phosphate-buffered saline (PBS). After the cells attached (>3 hours), the HPM was replaced by hepatocyte basal medium media+hepatocyte culture medium Single Quots (Clonetics; Lonza, Walkersville, MD).

Preparation of retroviral pseudoparticles. Murine leukemia virus (MLV)-based pseudotypes bearing vesicular stomatitis virus glycoproteins (VSV-Gs) or HCV E1-E2 proteins of the Con1, H77, or J6CF isolates were generated as described recently.[4]

HCV Pseudoparticle Infection of Primary Human Hepatocytes. Primary human hepatocyte cells infected with HCV pseudoparticles (HCVpp) were treated with increasing amounts of EGCG for 4 hours. After 48 hours, cells were analyzed on a fluorescence-activated cell sorter (FACS) (FACScalibur; BD). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

HCV Luciferase Replication and Infection Assay. Huh-7.5 cells were electroporated with 5 μg of the reporter virus genome, as previously described.[16] After 4 hours, different concentrations of EGCG were added to the cell-culture medium. HCV RNA replication was quantified by measuring luciferase activity. After 48 hours, supernatants were collected, filtered through 0.45-μm pore-size filters, and used to infect naïve Huh-7.5 target cells.

Detection of HCV Receptor Expression by Flow Cytometry and Western Blotting. Huh-7.5 cells were treated with increasing amounts of EGCG for 4 hours. After incubation with EGCG, cells were stained with antibodies for flow cytometry and western blotting, as previously described.[4]

Iodixanol Density-Gradient Fractionation. Density-gradient centrifugation was performed as described recently.[17]

Cell-to-cell spread assay. To measure HCV infection by direct cell-to-cell transmission between adjacent cells, we performed an agarose overlay assay in the presence or absence of EGCG, as described recently.[18]

^{35}S Labeling. Huh-7.5 cells seeded in 100-mm dishes were infected with HCV JFH-1 (multiplicity of infection [MOI]: 0.13 focus-forming units [ffu]/cell). Inocula were removed after 4 hours at 37°C, and the infected cells were washed twice with PBS before the addition of DMEM/10% FBS. Cells were methionine-starved 3 hours later by replacing the media with methionine-free DMEM/10% FBS. After 2 hours of methionine starvation, cells were washed twice with warm PBS and overlaid with 4 mL of methionine-free DMEM/10% FBS, supplemented with 42 μCi/mL of ^{35}S-methionine (PerkinElmer, Waltham, MA). Supernatants were recovered approximately 48 hours later and centrifuged at 1,200 rpm at 4°C for 5 minutes to pellet cell debris. The supernatant was filtered through a 0.22-μm filter and concentrated using Amicon 100-K molecular-weight cut-off filters (Millipore, Bedford, MA). The resulting virus stock was titrated using the focus-forming assay and stored at −80°C. ^{35}S incorporation was tested using a Beckman Coulter LS 6500 scintillation counter (Beckman Coulter, Inc., Brea, CA). HCV virions were labeled to a calculated 199 cpm/ffu.

Binding Assay. [^{35}S]-methionine-labeled HCV virions were exposed to EGCG, EC, or dimethyl sulfoxide (DMSO) vehicle for 10 minutes at 37°C. Next, 6×10^5 cpm (3×10^3 ffu) of ^{35}S-Met-HCV was adsorbed onto nearly confluent monolayers of Huh-7.5 cells for 1 hour at 4°C before washing four times with ice-cold PBS. Radioactivity attached to cells after the washes was measured using a Beckman Coulter LS 6500 scintillation counter. Binding was calculated as counts per minute (cpm) bound to cells divided by total cpm, adjusted by background and expressed as a percentage. Percent binding was then expressed relative to binding of virions exposed to vehicle control.

Results

EGCG Inhibits HCVcc Entry but Has No Effect on HCV RNA Replication or Assembly and Release. To evaluate the effects of EGCG on HCV replication and infectivity, we used firefly luciferase reporter viruses (Luc-Jc1). Huh-7.5 cells were transfected with the reporter virus genome, and increasing doses of EGCG were added 4 hours later. Replication efficiency was assessed 48 hours after transfection using luciferase assays. No effect of EGCG on HCV RNA replication could be detected (Fig. 1A). However, EGCG present during infection of naïve Huh-7.5 cells with HCVcc (produced in the absence of EGCG) resulted in dose-dependent inhibition of infectivity (Fig. 1B). The half-maximal inhibitory concentration (IC_{50}) was

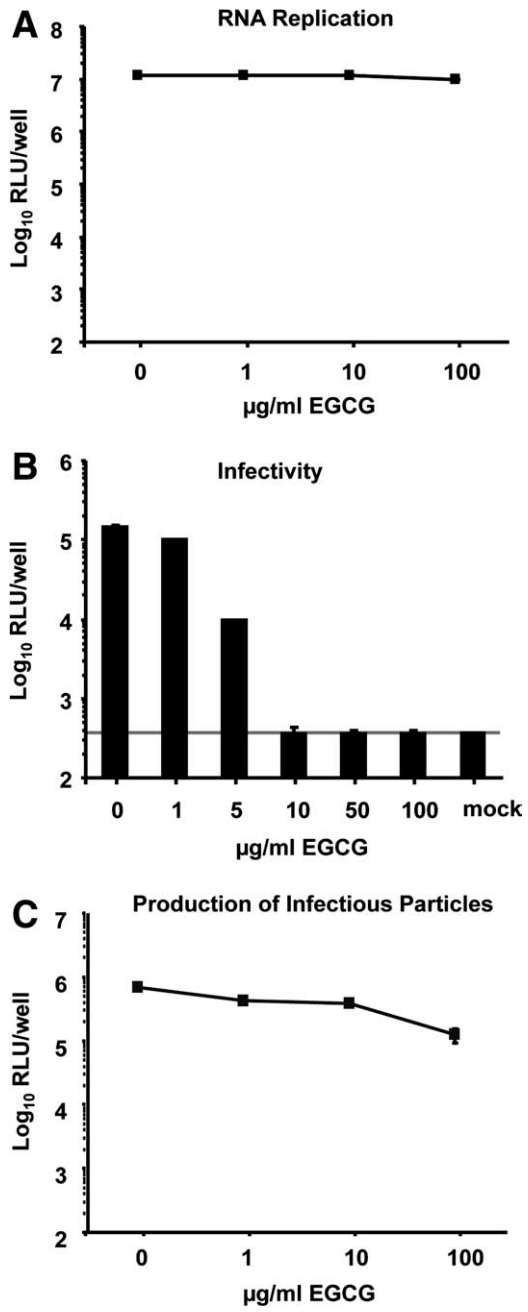

Fig. 1. The catechin, EGCG, reduces HCV infectivity in a dose-dependent manner. (A) Huh-7.5 cells were electroporated with the reporter virus, Luc-Jc1, and 4 hours later, increasing doses of EGCG were added. Replication efficiency was assessed 48 hours after transfection using luciferase assays. A representative experiment of three independent repetitions with standard deviation of the mean, too small to be seen at this scale, is shown. (B) Huh-7.5 cells were inoculated with Luc-Jc1 reporter viruses in the presence of increasing concentrations of EGCG. The inoculum was removed 4 hours later, then monolayers were washed three times with PBS and overlaid with fresh medium containing no EGCG. Infected cells were fixed 3 days later, and luciferase activity was determined. A representative experiment of three independent repetitions with standard deviation of the mean, too small to be seen at this scale, is shown. (C) Culture supernatant from the cells in (A) was used to inoculate naïve Huh-7.5 cells. Infected cells were fixed 3 days later, and their luciferase activity was determined. A representative experiment of three independent repetitions with standard deviation of the mean, too small to be seen at this scale, is shown.

approximately 2.5 µg/mL. At concentrations of 10 µg/mL, luciferase activity was at the level of that in mock-infected cells. In parallel, the supernatant from electroporated and EGCG-treated cells from Fig. 1A was used to inoculate naïve cells to detect a possible influence of the drug on virus production (Fig. 1C). However, only a very slight reduction at the highest levels of EGCG was observed. These data suggest that EGCG is an HCV entry inhibitor that does not affect RNA replication or release of infectious particles.

HCV inhibition Is Unique to EGCG and Not Shared by Other Green Tea Catechins. EGCG is the major component of the polyphenolic fraction of green tea (up to 50%). To test whether other catechins in green tea would have an influence on HCV entry, we next assessed the effect of EGC, EC, and ECG on HCVcc infection. Huh-7.5 cells were electroporated with the reporter virus genome, and 4 hours later, increasing doses of the different compounds were added. EGC, EC, and ECG had no obvious inhibitory activity on HCVcc at the highest concentration used, in contrast to EGCG (Fig. 2). Unlike what has been reported for other viruses, therefore, inhibition of HCV entry seems to be unique to EGCG and not shared by other green tea catechins.

EGCG Inhibits Cell Entry Mediated by Diverse Viral Envelope Proteins and in Primary Hepatocytes. To further test whether EGCG targets HCV entry, we next used HCVpp. These are retroviral cores carrying HCV glycoproteins in their envelope. In this

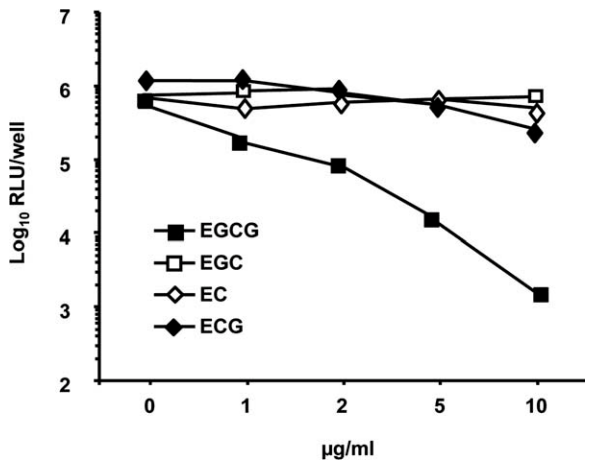

Fig. 2. EGCG is the most active catechine at inhibiting HCV infectivity. Huh-7.5 cells were inoculated with Luc-Jc1 reporter viruses in the presence of increasing concentrations of EGCG, EGC, EC, or ECG. The inoculum was removed 4 hours later, then monolayers were washed 3 times with PBS and overlaid with fresh medium containing drug. Infected cells were fixed 3 days later, and luciferase acitivity was determined. A representative experiment of three independent repetitions with standard deviation of the mean, too small to be seen at this scale, is shown.

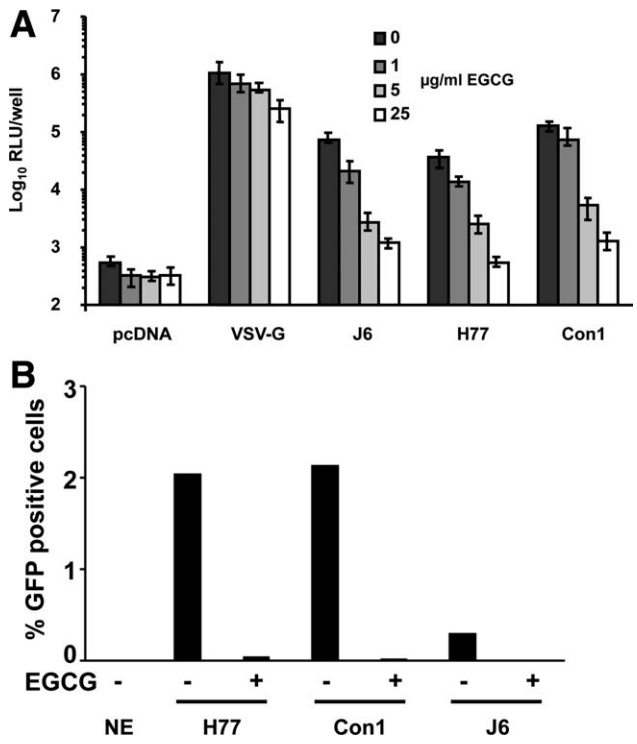

Fig. 3. EGCG specifically inhibits HCV entry into Huh-7.5 cells and primary human hepatocytes. (A) Huh-7.5 cells were infected with HCV pseudoparticles of genotype 2 (J6), 1a (H77), or 1b (Con1) in the presence of different concentrations of EGCG during infection. The inoculum was removed 4 hours later, then monolayers were washed and overlaid with fresh medium containing drug. Infected cells were fixed 3 days later, and luciferase acitivity was determined. A representative experiment of three independent repetitions with standard deviations of the means is shown. (B) Primary human hepatocytes were infected with HCV pseudoparticles, as described in (A). EGCG was added during infection at a concentration of 10 μg/mL. A representative experiment of three independent repetitions is shown.

context, only the early steps of virus entry (i.e., virus binding, uptake, and fusion) are HCV specific, whereas all later steps are dependent on retroviral nucleocapsid elements. Using this approach, EGCG inhibited cell entry mediated by HCV glycoproteins and also—to a far lesser extent—retroviral particles pseudotyped with the VSV-G (Fig. 3A). Infectivities of HCVpp carrying E1E2 proteins of the strains, J6CF (genotype 2), H77 (genotype 1a), and Con1 (genoytpe1b), were similarly reduced, indicating that HCV inhibition by this flavonoid is not genotype or isolate specific. Although human HCC-derived cell lines allow the study of the complete HCV replication cycle, these cells are phenotypically and functionally very different from hepatocytes *in vivo*. Primary human hepatocytes (PHHs) may more closely resemble the hepatocytes that are the main reservoir for HCV within the infected host. As PHHs do not support robust replication of HCV genomes *in vitro*, PHHs were infected in the presence or absence of EGCG with HCVpp of strains J6CF, Con1, or H77 carrying a green fluorescent protein reporter gene, as described above. Entry of all isolates into PHH was strongly inhibited by 10 μg/mL of EGCG, as tested by FACS (Fig. 3B). Thus, EGCG is likely to also block HCV entry into its natural target cells.

EGCG Has No Influence on HCV Entry Factor Expression and Virus Particle Density. To analyze the mechanism by which EGCG inhibits HCV entry, we assessed the expression of the known essential HCV (co-)receptors, cluster of differentiation (CD)81, scavenger receptor class B type 1 (SR-BI), claudin-1

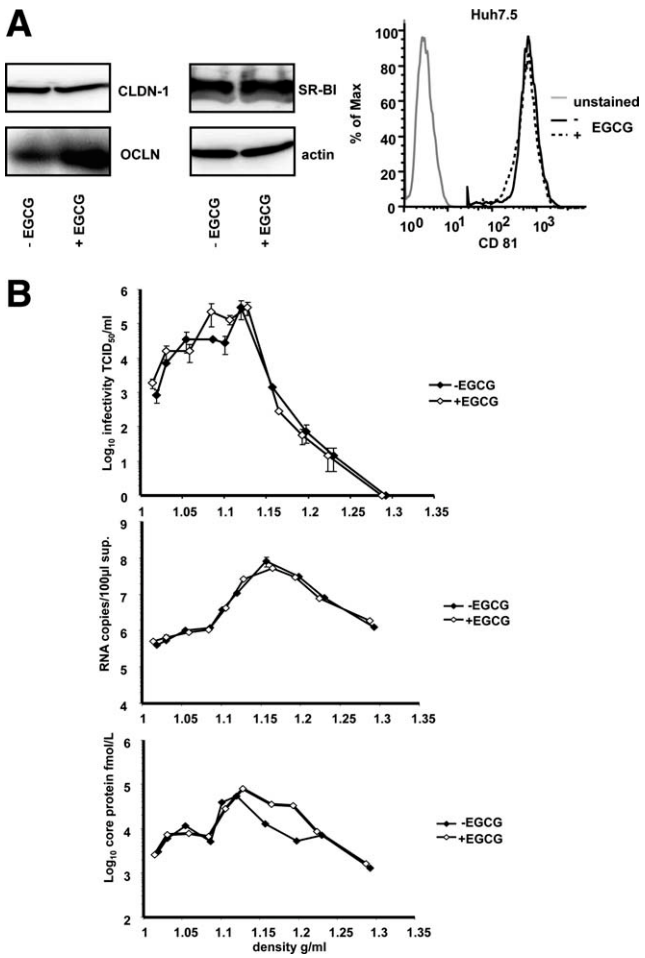

Fig. 4. EGCG does not change expression levels of cellular entry factors or the density profile of HCV virions. (A) Huh-7.5 cells were treated with EGCG for 4 hours and levels of CLDN-1, OCLN, and SR-BI were measured by western blotting, whereas CD81 surface expression was measured by FACS. (B) Huh-7.5 cells were electroporated with Jc1, and 10 μg/mL of EGCG was added 4 hours later. Cell-culture supernatants were harvested 48 hours later, and iodixanol step-gradient centrifugation was performed. HCV infectivity, HCV RNA, and core protein were determined for each of 10 gradient fractions harvested from the bottom. Values are plotted against the density of the respective fraction, as measured by refractometry. A representative experiment of three independent repetitions with standard deviations of the means is shown.

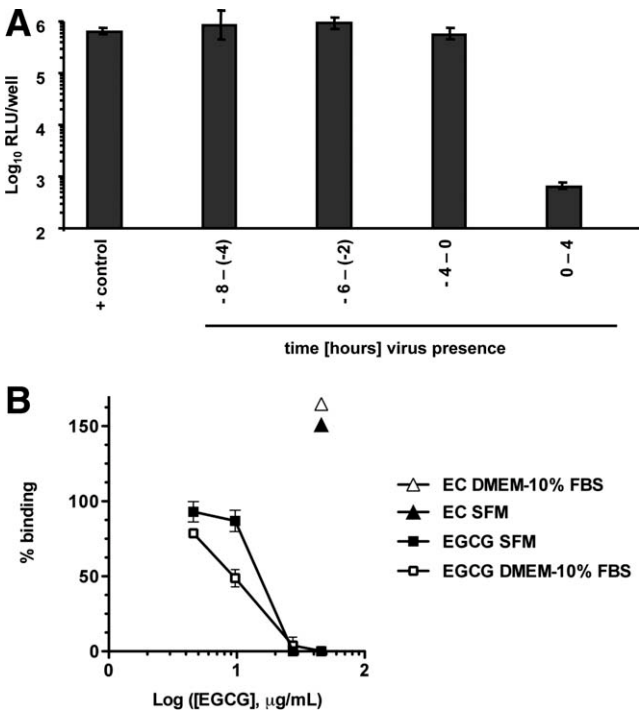

Fig. 5. EGCG inhibits HCV virion attachment to target cells. (A) Huh-7.5 cells were treated with 10 μg/mL of EGCG at different time points before or during infection with Luc-Jc1, with time point "0" being the time when virus was added to the cells. After each incubation time frame, the inoculum was removed, cells were washed, and new media was added. After 72 hours, infected cells were fixed and luciferase activity was determined. A representative experiment of three independent repetitions with standard deviations of the means is shown. (B) ^{35}S-HCV JFH-1 preexposed to EGCG, EC, or DMSO vehicle for 10 minutes at 37°C was adsorbed onto near-confluent Huh-7.5 cell monolayers for 1 hour at 4°C. Cells were then washed four times with ice-cold PBS. Binding was calculated by cpm bound to cells after the washes divided by total cpm, adjusted by background. Percent binding is expressed relative to the binding of vehicle-treated virions. Mean values and ranges of two independent experiments are presented.

(CLDN1), and occludin (OCLN). Huh-7.5 cells were treated with EGCG at 10 μg/mL for 4 hours, then (co-)receptor of SR-BI, CLDN1, and OCLN expression was assessed by western blotting or flow cytometry (Fig. 4A). The expression levels of all four coreceptors were unaltered, indicating that EGCG does not act through their down-regulation.

To test whether EGCG would result in an alteration of HCV-lipoprotein association, we centrifuged HCVcc particles through iodixanol density gradients in the presence or absence of the compound. HCVcc displayed a typical broad-density distribution, as evaluated by viral RNA, proteins, or infectivity. This broad density of HCV particles is thought to be the result of an inhomogeneous association of virions with lipoproteins. This inhomogeneous association has, in turn, been suggested to modulate infectivity and the ability to evade humoral immune responses *in vivo*. To test whether EGCG would act through a perturbation of lipoprotein association or another modification of the viral particle resulting in an altered density profile, we produced HCVcc in the presence of EGCG. Cell-culture supernatants were harvested 48 hours later, and iodixanol step-gradient centrifugation was performed. Fractions were collected and assayed for viral infectivity, HCV RNA, and HCV core protein (Fig. 4B). No major changes in density distribution were observed, indicating that EGCG acts on HCV through a mechanism that does not grossly alter particle density or lipoprotein association. Alternatively, EGCG may impede HCV cell entry by acting on the host cell.

EGCG Does Not Act on Target Cells but Inhibits HCV Attachment. To probe whether the inhibitory effect of EGCG would be caused by an alteration of the target cell rendering it HCV resistant, we administered EGCG during different time windows before or during the early phase of infection. Application and removal of EGCG before inoculation with virus did not reduce HCV infection, whereas treatment with EGCG directly during inoculation strongly inhibited HCV infectivity (Fig. 5A).

The inhibitory effect of EGCG on HCV infectivity could have resulted from the inhibition of virion attachment. To test for this possibility, ^{35}S-methionine-labeled HCV virions preexposed to EGCG or EC were adsorbed onto cells at 4°C to allow binding, but not fusion. Attachment was evaluated in the absence or presence of serum, used to block nonspecific binding. EGCG inhibited binding of HCV to cells in a dose-dependent manner, with an IC_{50} value of 9.7 μg/mL in the presence of 10% serum or 17.2 μg/mL in the absence of serum (Fig. 5B). In contrast, the related catechin, EC, did not inhibit HCV attachment to cells (Fig. 5B). In conclusion, EGCG inhibits HCV infectivity by inhibiting its primary attachment to cells.

Inhibition of HCV Cell-to-Cell Transmission by EGCG. It has been reported that HCV can also be transmitted via cell-to-cell spread. This transmission may be important *in vivo* and was reported to be refractory to neutralization by E2 monoclonal antibodies and occur in a CD81-independent manner. Therefore, we tested whether EGCG would also block cell-to-cell transmission of HCV in tissue culture. Huh-7.5 cells were infected with HCVcc (H77/JFH1 chimera) at a high MOI, and we confirmed that >99% of the cells were HCV positive by immunofluorescence against NS5A (data not shown). These "donor cells" were cocultured with Huh-7.5 "acceptor" cells that carry a reporter protein allowing detection of HCV infection

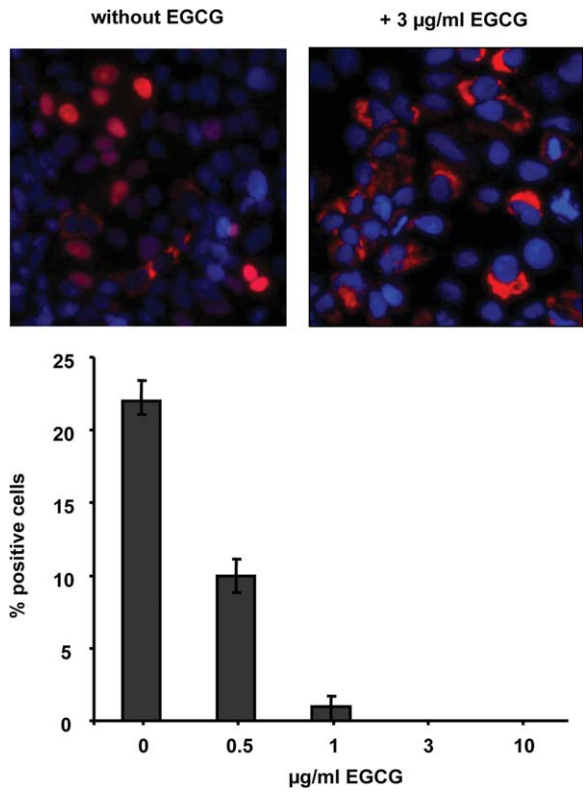

Fig. 6. EGCG inhibits HCV cell-to-cell spread. A complete H77/JFH1-infected population of nonfluorescent Huh-7.5 donor cells was put in coculture, with uninfected Huh-7.5 target cells harboring a tagRFP-NLS-IPS reporter[19] in the presence or absence of EGCG, and was overlaid with 1% agarose. Fluorescence is localized in the cytoplasma in uninfected cells (tagRFP). Upon HCV infection, tagRFP-NLS-IPS is cleaved and the fluorescent reporter relocates to the nucleus. Nuclei were costained with 4′,6-diamidino-2-phenylindole. Percentage of infected cells in increasing amounts of EGCG is presented, as evaluted by nuclear localization of tagRFP at 96 hours of coculture. A representative experiment of two independent repetitions with mean values and ranges are presented.

at a single cell level by relocation of a red fluorescent protein (tagRFP) from a mitochondrial (i.e., uninfected) to a nuclear (i.e., infected) localization.[19] Cocultured cells were overlaid with media containing increasing amounts of EGCG and 1% agarose, as reported previously, to prevent cell-free diffusion of virus.[20] Cell-to-cell transmission was inhibited by EGCG (Fig. 6. Not a single secondary infection event could be recorded at 3 μg/mL of EGCG. These results demonstrate that EGCG prevents HCV cell-to-cell spread.

Finally, we tested whether EGCG could be combined with other antiviral compounds targeting HCV replication in an IFN-free regimen. We used INN, a drug targeting directly the HCV protease (NS3/4A) and CyA, which inhibits RNA replication via the HCV cofactor, cyclophilin A. We titrated both drugs in the presence of EGCG and could observe a strong, additive inhibition of HCV infection at low concentrations of INN and CyA (Supporting Fig. 1). These data indicate that EGCG could efficiently be used in combination therapy.

Discussion

In this study, we identified EGCG, a natural compound contained in green tea, as an inhibitor of HCV entry into target cells and cell-to-cell spread between neighboring cells. The effect is unique to EGCG and not shared by other green tea catechins. It was readily detectable for all HCV genotypes tested and in hepatoma cell lines as well as PHH. Moreover, we demonstrated that EGCG acts by blocking viral attachment to target cells (i.e., the initial step of the cell entry process), whereas it does not affect other replication cycle stages. The biophysical properties of the virions and the receptor expression levels on the target cells also remained unaltered by EGCG.

HCV entry is essential for initiation, spread, and maintenance of virus infection and represents an interesting target for antiviral therapy. With the NS3/4A protease inhibitors, INN and telaprevir, the first anti-HCV drugs beyond IFN and ribavirin are reaching the market in 2011. Yet, like all other anti-HCV compounds in late-stage clinical development, they target the inhibition of viral RNA replication. In the setting of liver transplantation for HCV-associated end-stage liver disease, moreover, the ability to block cell entry would help in minimizing the currently universal reinfection of the donor liver by virions in the blood.

Other agents have previously been reported to inhibit HCV cell entry through various mechanisms. The lectin, cyanovirin-N, interacts with high-mannose oligosaccharides on viral envelope glycoproteins and prevents its interaction with cellular receptor molecules, presumably CD81.[21] Oxidized low-density lipoproteins (oxLDL), a physiologically occurring subfraction of LDL, are thought to inhibit an SR-BI-mediated step in the HCV entry process.[22] Antibodies targeting the glycoproteins or cellular receptors, such as CD81, SR-BI, and CLDN1, have also been shown to block viral entry and control spread *in vitro* and *in vivo*.[23] The small molecule compound, ITX-5061, was described to disrupt the interaction of E2 and SR-BI and is currently entering a phase Ib study in humans.[24] Baldick et al. have recently identified a compound (El-1) that inhibits a postattachment step of the cell entry process, but which appears less active against non-1 genotypes.[20] A recent study by Lupberger et al. identified the epidermal growth factor

receptor (EGF-R) as a host factor required for HCV entry and suggested the EGF-R inhibitor, erlotinib, as a possible anti-HCV agent.[25] Also, small molecule inhibitors of viral entry, which act on envelope lipids to prevent the formation of the negative curvature required for fusion, inhibit HCV JFH-1 infectivity to Huh-7.5 cells in culture.[26] However, compared to these other inhibitors of HCV entry, EGCG is of particular interest, because it is active *in vitro* against all HCV genotypes tested, known to be innocuous in humans, readily available, and cheap. In fact, EGCG and various green tea preparations are available as an over-the-counter remedy in many countries.

EGCG is water soluble in green tea produced from the leaves of the plant, *Camellia sinensis*. The major active ingredients of green tea are polyphenolic compounds, known as catechins. The catechines include EGCG, EGC, ECG, and EC, of which EGCG accounts for approximately 50% of the total green tea catechins. Interestingly, EC is inactive against HCV and lacks the gallic acid ester moiety of EGCG, as well as one additional phenolic hydroxyl group. Thus, these functional groups, therefore, contribute to the ability of EGCG to inhibit HCV attachment and infectivity.

In clinical studies with healthy human volunteers, it could be shown that EGCG is safe and very well tolerated with oral doses of 800 mg of EGCG per day over 4 weeks, which equals approximately 8-16 cups of green tea once a day.[27] Plasma concentration ranged from 0.13 to 3.4 μg/mL, which reaches the IC_{50} value of EGCG that we determined here (2.5 μg/mL), but would probably not be high enough to eliminate HCV completely. However, the bioavailability of EGCG can be increased by peracetylation, and further studies are required to determine the tissue distribution and the *in vivo* potency of the molecule against HCV.

Using radioactively labeled HCV, we showed that EGCG targets the very first step of the HCV cell entry process (i.e., attachment). Later entry steps, such as viral receptor interactions, endocytosis, or membrane fusion, appear not to be directly affected. Accordingly, viral association with lipoproteins and the HCV (co-)receptor expression levels on the host cell were not altered by EGCG treatment. Importantly, cell-to-cell HCV spread, which may be critical *in vivo*, was also inhibited by EGCG treatment.

Based on work in a cell-free system, it was previously suggested that EGCG also inhibits the essential HCV NS3/4A serine protease, but this inhibitory effect has not been validated in an HCV replication setting.[28] In our hands, HCV replication and assembly of full-length HCV genomes were unaffected by EGCG.

EGCG and the other green tea catechins have been reported to be active against viruses other than HCV, and different mechanisms of action appear to be involved. For influenza A virus, Song et al. proposed that EGCG and ECG are potent inhibitors of multiple steps of the viral life cycle, including hemagglutination, neuraminidase activity, and viral RNA synthesis.[12] In the case of hepatitis B virus, an inhibition of viral DNA synthesis has been proposed.[14]

In summary, the green tea molecule, EGCG, potently inhibits HCV entry independent of the genotype and in primary human hepatocytes by blocking viral attachment. This novel inhibitor may provide a new approach to prevent HCV infection, especially in the setting of liver transplantation of chronically infected HCV patients.

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