

Fig. 4. E6AP-dependent degradation of annexin A1 protein. A: HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 µg of pCAG-annexin A1-FLAG plus 4 µg of empty vector, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The cells were treated with 50 µg/ml CHX at 44 h after transfection. Cell extracts were collected at 0, 3, 6, and 9 h after treatment with CHX, followed by immunoblotting. Data are representative of three independent experimental determinations. B: Knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous annexin A1 in HEK 293T cells. HEK 293T cells (3×10^5 cells/6-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or scramble negative control). Two sets of siRNAs (siE6AP-1 and siCont-1, siE6AP-2 and siCont-2) were used as described in Materials and Methods Section. The cells were harvested at 120 h after siRNA transfection. The relative levels of protein expression were quantitated using the NIH Image 1.62 program and are indicated below in the respective lanes. Data are representative of three independent experimental determinations.

results indicate that E6AP binds annexin A1 in a Ca^{2+} -dependent manner.

UBIQUITYLATION OF ANNEXIN A1 BY E6AP IS Ca^{2+} -DEPENDENT

The HECT-type ubiquitin ligases transfer ubiquitin molecules to the substrates through direct interaction. Therefore, the E6AP-annexin A1 interaction is considered to be necessary for E6AP-mediated annexin A1 ubiquitylation. To determine if the molecular interaction is required for E6AP-mediated annexin A1 ubiquitylation, we performed *in vitro* ubiquitylation assay in the presence or absence of 1 mM $CaCl_2$. When *in vitro* ubiquitylation reaction was carried out in the presence of Ca^{2+} , the higher-molecular-weight species of GST-annexin A1 were detected with anti-GST PAb (Fig. 6C, lane 2), indicating that annexin A1 is polyubiquitylated by E6AP *in vitro*. However, no ubiquitylation signal was detected when the ubiquitylation reaction was carried out in the absence of Ca^{2+} (Fig. 6C, lane 1), indicating that the E6AP-annexin A1 interaction is required for E6AP-mediated annexin A1 ubiquitylation.

To further investigate whether the ubiquitylation of annexin A1 is dependent on the presence of Ca^{2+} , we examined the effects of EGTA on the E6AP-mediated ubiquitylation of annexin A1. Polyubiquitin chains were synthesized even in the presence of a high concentration of EGTA (Fig. 6D), indicating that E6AP was active even in the

presence of EGTA. However, increasing amounts of EGTA resulted in decreases in the ubiquitylation of annexin A1 (Fig. 6E), suggesting that chelating the Ca^{2+} in the reaction mixture with EGTA inhibits the ubiquitylation of annexin A1. These findings suggest that the ubiquitylation of annexin A1 by E6AP is dependent on the presence of Ca^{2+} .

E6AP-BINDING DOMAIN FOR ANNEXIN A1 PROTEIN

To map the E6AP-binding domain on annexin A1 protein, GST pull-down assays were performed using a panel of annexin A1 deletion mutants expressed as GST-fusion proteins. Figure 7A shows a schematic representation of annexin A1 and known motifs in annexin A1. A series of deletion mutants of annexin A1 as GST fusion proteins (Fig. 7A) were expressed in *E. coli*. Purified MEF-E6AP was used to determine E6AP-binding domain. GST pull-down assays revealed that the core domain of annexin A1 (42-346), but not the N-terminal tail of annexin A1 (1-41), bound to E6AP (Fig. 7B, lanes 4 and 3). GST pull-down assays also showed that annexin A1 (114-274) and annexin A1 (196-346), but not annexin A1 (42-195), were able to bind to E6AP (Fig. 7B, lanes 5-7). As shown in Fig. 7C, GST-annexin A1 (196-274) bound to E6AP. These findings suggest that annexin repeat domain III is important for E6AP binding.

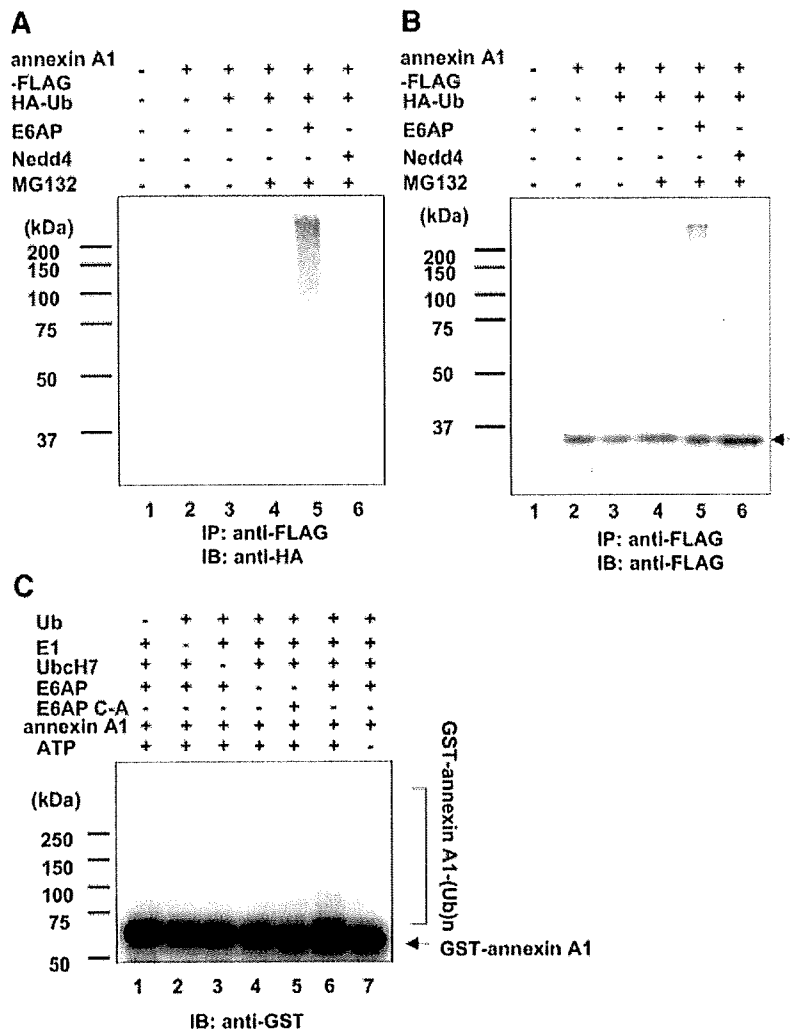


Fig. 5. E6AP-dependent ubiquitylation of annexin A1 protein in vivo and in vitro. HEK 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μ g of pCAG annexin-FLAG together with 2 μ g of plasmid encoding E6AP as indicated. Each transfection also included 2 μ g of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anti-FLAG PAb (B). The Western blot shows the presence of a ubiquitin smear. The arrow indicates annexin-FLAG. IB, immunoblot; IP, immunoprecipitation. C: In vitro ubiquitylation of annexin A1 by E6AP. For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40- μ l volumes containing each component as indicated. The reaction mixture is described in the Experimental Procedures. The reaction mixture contained 1 mM CaCl_2 . The reaction was terminated by addition of SDS-PAGE loading buffer and followed by immunoblotting with anti-GST PAb. The arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets.

DISCUSSION

In the present study, we have identified annexin A1 as a novel substrate for E6AP using four lines of evidence: (1) E6AP bound to annexin A1 in vivo and in vitro; (2) overexpression of E6AP enhanced proteasomal degradation of annexin A1 in vivo; (3) knockdown of endogenous E6AP by siRNA resulted in the accumulation of endogenous annexin A1 in vivo; and (4) E6AP enhanced the polyubiquitylation of annexin A1 in vivo and in vitro. These results provide evidence that E6AP mediates the ubiquitylation and proteasomal degradation of annexin A1. We have shown that E6AP bound to annexin A1 only in the presence of Ca^{2+} and that these interactions were enhanced by increasing concentrations

of Ca^{2+} . Annexin A1 was polyubiquitylated by E6AP only in the presence of Ca^{2+} . Chelating Ca^{2+} with EGTA inhibited E6AP-mediated polyubiquitylation of annexin A1. The E6AP-binding domain on annexin A1 was mapped to the core domain, especially the annexin repeat domain III. These results suggest that the conformational change of annexin A1 induced by Ca^{2+} binding allows E6AP to bind to annexin repeat domain III of annexin A1 and to mediate its ubiquitylation and degradation.

Post-translational modifications, such as Ca^{2+} binding, phosphorylation, and lipidation, have roles in the regulation of annexin A1. Solito et al. [2006] showed that the translocation of annexin A1 from the cytoplasm to the cell surface is regulated by phosphorylation and lipidation. Annexin A1 is phosphorylated by several

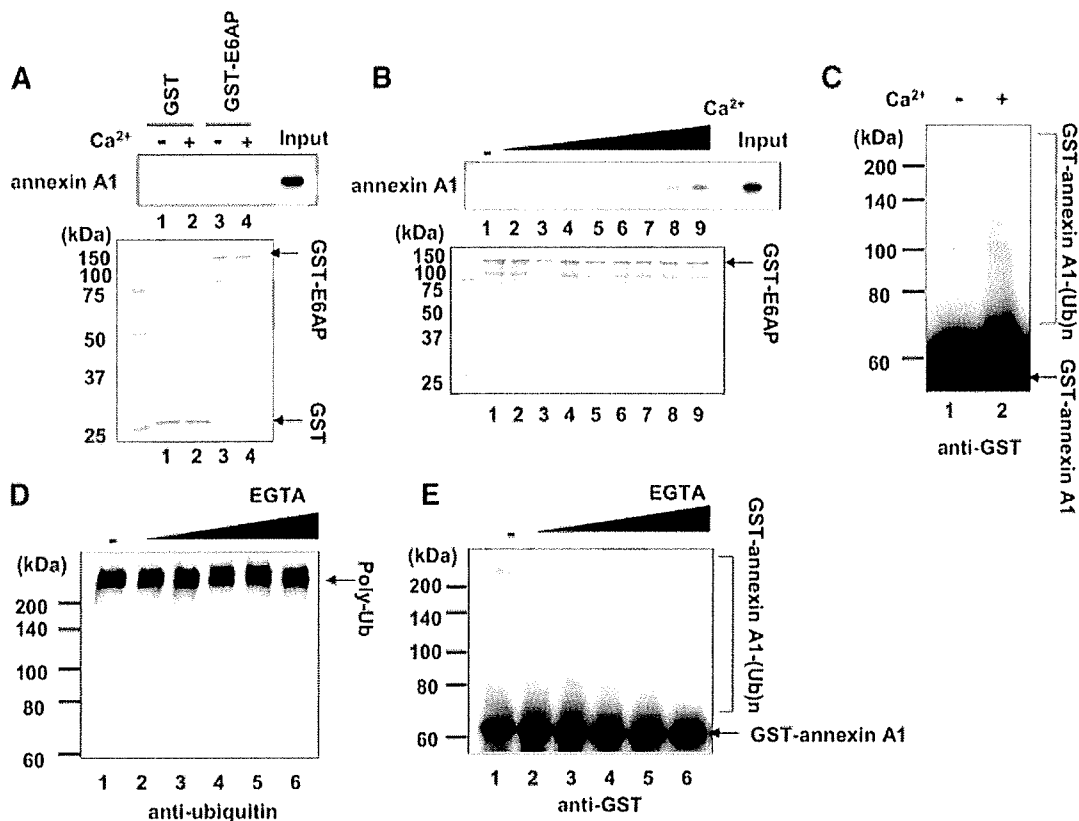


Fig. 6. E6AP mediates ubiquitylation of annexin A1 in a Ca^{2+} -dependent manner. **A:** In vitro binding of annexin A1 and E6AP. Immobilized GST-E6AP or GST alone was incubated with purified His-annexin A1 in the presence or absence of 1 mM CaCl_2 in the binding solution. Immunoblotting to detect bound annexin A1 was performed using anti-annexin A1 antibody. **B:** Ca^{2+} -dependent interaction between annexin A1 and E6AP. The GST pull-down assays described in (A) were repeated in the presence of increasing concentrations of CaCl_2 in the binding solution as follows: lane 1 (0 μM), 2 (10 μM), 3 (100 μM), 4 (250 μM), 5 (500 μM), 6 (750 μM), 7 (1 mM), 8 (2.5 mM), and 9 (5 mM). **C:** For in vitro ubiquitylation of annexin A1 protein, purified GST-annexin A1 was used as a substrate. Assays were done in 40- μl volumes in the presence or absence of 1 mM CaCl_2 . The reaction mixture is described in Materials and Methods Section. The reaction was terminated by addition of SDS-PAGE loading buffer and followed by immunoblotting with anti-GST PAb. Arrow indicates GST-annexin A1. Ubiquitylated species of GST-annexin A1 proteins are marked by brackets. **D,E:** The in vitro ubiquitylation assays were performed in the presence of various concentrations of EGTA in the reaction mixture containing 1 mM CaCl_2 . The concentrations of EGTA were as follows: lane 1 (0 mM), 2 (0.1 mM), 3 (0.5 mM), 4 (1 mM), 5 (5 mM), and 6 (10 mM). **D:** Immunoblotting to detect whole polyubiquitylated proteins with anti-ubiquitin MAb. **E:** Immunoblotting to detect polyubiquitylated GST-annexin A1 with anti-GST PAb.

protein kinases, such as epidermal growth factor receptor protein kinase, protein kinase C, and hepatocyte growth factor receptor kinase to mediate proliferation [Lim and Pervaiz, 2007], suggesting that phosphorylation plays some roles in the regulation of annexin A1 function. The findings presented in this study suggest that the ubiquitin-proteasome pathway plays a role in the regulation of annexin A1 function. Our data also suggest that E6AP preferentially recognizes the Ca^{2+} -binding form of annexin A1 and targets it for proteasomal degradation. The main biological property of annexin A1 is the binding to the phospholipid membrane in a Ca^{2+} -dependent manner [Lim and Pervaiz, 2007]. X-ray crystallography studies of annexin A1 have suggested that a calcium-driven conformational switch of the N-terminal and core domains of annexin A1 involves the membrane aggregation properties of annexin A1 [Rosengarth et al., 2001; Rosengarth and Luecke, 2003]. It will be intriguing to examine the role of E6AP in membrane aggregation. Further investigations will be

required to elucidate the role of E6AP in the regulation of annexin A1 functions.

Targeting of a substrate via the ubiquitin system involves specific binding of the protein to the appropriate E3 ubiquitin ligase. There are several modes for specific substrate recognition, such as (1) NH₂-terminal residue (N-end rule pathway), (2) allosteric activation, (3) recognition of phosphorylated substrate, (4) phosphorylation of E3, (5) phosphorylation of both the ligase and its substrate, (6) recognition in trans via an ancillary protein, (7) abnormal/mutated/misfolded proteins, and (8) recognition via hydroxylated proline [Glickman and Ciechanover, 2002]. E6AP specifically recognizes active forms of Blk, indicating that tyrosine phosphorylation of the regulatory tyrosine of Blk plays a role in specific substrate recognition [Oda et al., 1999]. Here we propose a novel mechanism of specific substrate recognition in the ubiquitin system, in which E6AP recognizes annexin A1 via a Ca^{2+} -induced conformational change. E6AP plays a direct catalytic role in the

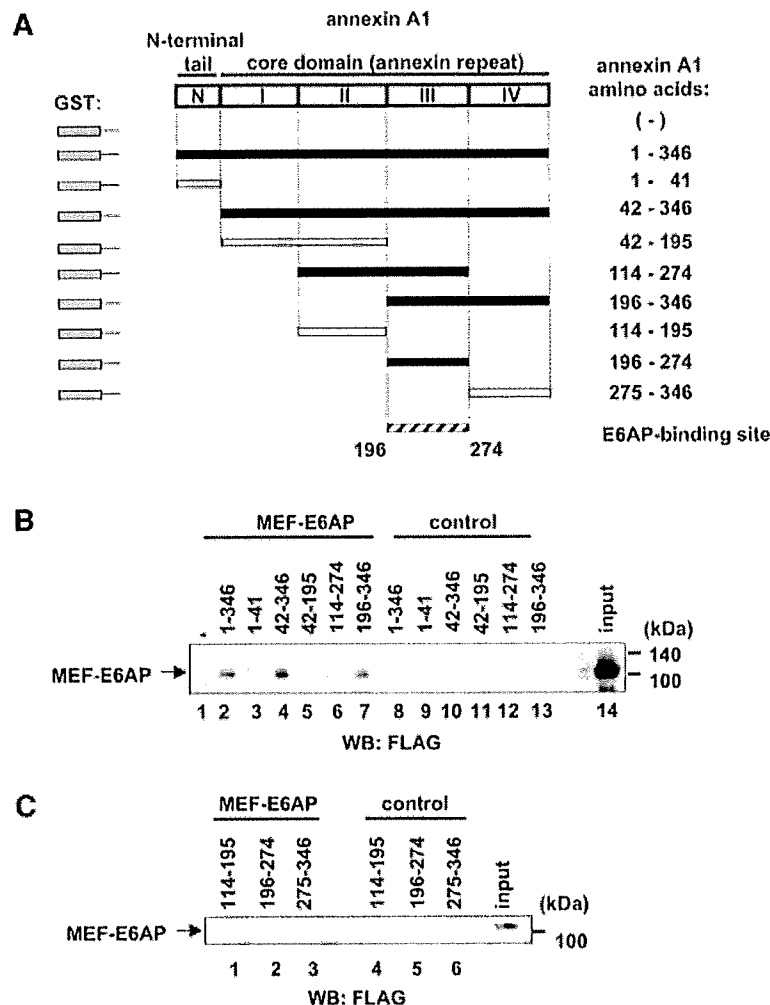


Fig. 7. Mapping of the E6AP-binding domain for annexin A1 protein. A: Structure of annexin A1. Shown is a schematic representation of the region of annexin A1 protein. N-terminal tail (aa 1–41), core domain (aa 42–346), and annexin repeat domains I (aa 42–113), II (aa 114–195), III (aa 196–274), and IV (aa 275–346) are shown. Schematic representation of GST-annexin A1 proteins. GST proteins contain the annexin A1 amino acids indicated to the right. The shaded box in each represents the GST sequence. Closed boxes represent proteins that are bound specifically to MEF-E6AP, and open boxes represent those that are not bound. B,C: In vitro binding of MEF-E6AP to GST-annexin A1 proteins. Purified recombinant MEF-E6AP was assayed for association with GST (-) or the GST-annexin A1 proteins using the binding buffer with 1 mM CaCl_2 . GST pull-down was performed to assay for the association of E6AP with annexin A1. Control experiments were performed without MEF-E6AP. The association of MEF-E6AP was detected by immunoblotting with anti-FLAG MAb.

final attachment of ubiquitin to substrate proteins. Our findings suggest that Ca^{2+} -induced conformational change of annexin A1 may function as a degradation signal for annexin A1.

Ubiquitylated annexin A2 is enriched in the cytoskeleton fraction of mouse Krebs II cells [Lauvrak et al., 2005]. It remains unclear whether the ubiquitylated annexin A2 is degraded by proteasome. The apical membrane localization of Nedd4, a member of HECT-type ubiquitin ligases, is mediated by an association of its C2 domain with the apically targeted annexin XIIIb [Plant et al., 2000]. However, it is unknown whether annexin XIIIb is a substrate of Nedd4. To our knowledge, this is the first study to identify a specific E3 ubiquitin ligase for the ubiquitylation of an annexin family protein. All annexins share a core domain composed of four similar repeats, each approximately 70 amino acids long. Each repeat is

composed of five α helices and usually contains a characteristic type-2 motif for binding calcium ions with the sequence GxGT-[38 residues]-D/E [Moss and Morgan, 2004]. The core domains of most vertebrate annexins reveal conservation of their secondary and tertiary structures despite the presence of only 45–55% amino-acid identity among individual annexins [Moss and Morgan, 2004]. It will be required to investigate whether other annexins are regulated by E6AP or other E3 ubiquitin ligases.

E6AP is hijacked by the HPV16E6 to target the tumor suppressor p53 in cervical cancer. Moreover, E6AP is mutated in Angelman syndrome and mediates ubiquitin-dependent degradation of HCV core protein, suggesting that E6AP plays important roles in sporadic and hereditary human diseases including cancer, neurological disorders, and infectious diseases [Kishino et al., 1997; Scheffner

and Staub, 2007; Shirakura et al., 2007]. Physical and functional association of E6AP with viral proteins, such as HPV16E6 [Huibregtse et al., 1993b] and HCV core protein [Shirakura et al., 2007], have been demonstrated. It is possible that the viral proteins redirect E6AP away from annexin A1, increasing the stability of annexin A1, and thereby contributing to viral pathogenesis. It would be interesting to investigate whether these viral proteins affect E6AP-dependent degradation of annexin A1. The association of E6AP with the viral protein (HPV16E6 or HCV core protein) could provide a feasible target for molecular approaches in the treatment of cervical cancer or HCV-related diseases.

In conclusion, we have demonstrated that E6AP interacts with annexin A1 protein and mediates its ubiquitin-dependent degradation. We propose that E6AP may play a role in regulating the diverse functions of annexin A1 protein. Identification of the specific E3 ubiquitin ligase may provide a link between the annexin family proteins and the ubiquitin-proteasome pathway. Elucidating the regulation of annexin A1 may provide a novel clue in the treatment of the E6AP-related diseases.

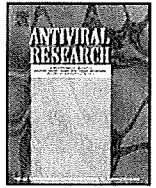
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Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR

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ABSTRACT

We devised a screening method for hepatitis C virus (HCV) inhibitors by exploiting the JFH1 viral culture system. The viral RNA released in the medium was adsorbed onto PCR plates, and real-time RT-PCR was performed by directly adding the one-step RT-PCR reaction mixture to the wells. The “tube-capture-RT-PCR” method obviates the need for labor-intensive RNA isolation and should allow high-throughput screening of HCV inhibitors. To substantiate the validity of the assay for drug screening, a pilot screen of an inhibitor library composed of 95 compounds was performed. In addition to the known inhibitors of HCV replication included in the library, the assay identified the PKC inhibitor bisindolylmaleimide I (BIM I) as an HCV replication inhibitor. BIM I was also effective in reducing the viral protein level in genotype 1b and 2a subgenomic replicon cells, indicating inhibition of HCV replication. Further assays revealed that a broad range of bisindolylmaleimides and indolocarbazoles inhibit HCV, but no correlation was found between the PKC inhibition pattern and anti-HCV activity. These series of compounds represent new classes of inhibitors that may warrant further development.

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1. Introduction

Hepatitis C virus, a major cause of chronic liver disease, has infected over 170 million people. The current mainstream anti-HCV therapy is a combination of interferon (IFN) and ribavirin. However, the therapy is not effective in approximately half of HCV-infected patients and has considerable side effects in many patients; thus, there is an urgent need for novel HCV therapies.

Various assays for HCV drug screening have been reported, many of which rely on HCV replicon systems. Although HCV replicon-based systems have greatly facilitated HCV research and drug discovery, these systems do not completely reflect the entire HCV life cycle and are not capable of identifying inhibitors of several important steps such as viral attachment, entry, and release. The recently introduced HCV cell culture systems (Wakita et al., 2005) should overcome these limitations and enable identification of inhibitors that would not be recognized by the replicon-based screens.

Here we describe a simple screening method for discovering anti-HCV drugs using the JFH1 viral culture system. Antiviral activ-

ity was determined by RT-PCR measurement of viral RNA released in the medium of infected cells. To increase efficiency, we devised a method that avoids tedious RNA isolation.

As a proof of concept, the method was used to evaluate a compound library and successfully confirmed the anti-HCV activity of cyclosporin A. In addition, a potent and selective PKC inhibitor, BIM I, was also identified as an anti-HCV agent. We found that other bisindolylmaleimides and indolocarbazoles also inhibit HCV, whereas anti-HCV activity was not associated with PKC inhibition. HCV inhibition by bisindolylmaleimides or indolocarbazoles has not been reported, and we expect that our assay will facilitate the identification of previously unrecognized HCV inhibitors. The bisindolylmaleimides and indolocarbazoles are already in clinical trials and may merit attention as HCV drug candidates.

2. Materials and methods

2.1. Cells and virus

Plasmid pJFH1, containing full-length cDNA of the JFH1 isolate, was used to generate HCV production in cell culture, as described elsewhere (Wakita et al., 2005), and the supernatant was passaged in Huh 7.5.1 cells. To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 days post-infection and stored at

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–80 °C until use. The infectious titers of the viruses were determined by immunofluorescence analysis of the infected Huh 7.5.1 cells using anti-core antibody (2H9). The infectious titers of the stocks were generally about 3×10^5 – 1×10^6 ffu/ml, corresponding to about 3×10^7 – 1×10^8 copies of JFH1 RNA/ml. A subgenomic replicon cell, clone 4-1, which harbors the genotype 2a HCV genome (Kato et al., 2003; Date et al., 2004) and clone 5-15, which harbors the genotype 1b HCV genome (Lohmann et al., 1999), were also cultured in Dulbecco's Modified Eagle's medium (DMEM) with fetal bovine serum (FBS).

2.2. Reagents

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. The PKC β isozyme selective inhibitor LY333531 (Ruboxistaurin) was from Alexis Corp. (Lausen, Switzerland). Other chemicals were purchased from Merck Calbiochem (Darmstadt, Germany). Interferon- α (IFN- α) was from PeproTech, Inc. (Princeton Business Park, Princeton, NJ).

2.3. Quantitative real-time RT-PCR

Huh 7.5.1 cells were seeded in 96-well plates at a density of 20,000 cells per well in a volume of 120 μ l. The next day, 15 μ l of test compounds was added and the cells were infected with 15 μ l of virus stock of HCV-JFH1 at a multiplicity of infection (MOI) of 0.01. After 5 days of culture, 100 μ l of medium was transferred to a PCR plate, incubated on ice for 30 min, centrifuged at 3500 rpm for 15 min, and then removed. Twenty microliters of One Step SYBR PrimeScript RT-PCR Kit reaction mixture (Takara-Bio Co., Otsu, Japan) was added into the PCR plate wells, and quantitative real-time PCR was performed using an ABI Prism 7000 sequence detector (PE Applied Biosystems, Foster City, CA). The primers used were 5'-GAGTGTCTACAGCCTCCAG-3' (nucleotides 97–116), and 5'-AGGCCTTTCGCAACCCA-3' (nucleotides 280–264) from the non-coding region of HCV-JFH1, at a concentration of 200 nM. Media from the control wells without drug were serially diluted to create a standard curve, which was used to determine the relative amount of HCV RNA in the media of HCV-infected cells treated with the compounds. Cell growth was monitored by MTT assay, as described previously (Fukazawa et al., 1995).

For further analysis of the drug effect and determination of the copy number of HCV RNA in medium and cells, HCV RNA was extracted from 140 μ l medium with the QIAamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany), and eluted with 60 μ l of elution buffer. Eight microliters of the viral RNA eluate was subjected to quantitative real-time PCR using Taqman EZ RT-PCR Core reagents (PE Applied Biosystems). The primers were 5'-CGGGAGAGCCATAGTGG (nucleotides 129–145) and 5'-AGTACCACAAGGCCTTTCG (nucleotides 289–271) at a concentration of 200 nM, and the Taqman probe was FAM-5'-CTGCGGAACCGGTGAGTACAC-3'-TAMRA (nucleotides 147–167) at a concentration of 300 nM (Takeuchi et al., 1999). Standard JFH1 RNA for measurement of copy number was transcribed from plasmid pSRG-JFH1-Luci, which was derived from pSRG-JFH1 (Kato et al., 2003), using the AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI). The transcribed RNA was purified and diluted with ribonuclease-free water containing yeast tRNA and 0.2% DTT, as previously described (Suzuki et al., 2005).

2.4. Western blotting

Cells were lysed with Radio-ImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5%

sodium deoxycholate, 1% NP-40, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 μ g/ml of each of antipain, pepstatin, and leupeptin, and centrifuged. The amount of protein in the supernatant was then measured. Cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies against core (2H9), NS5A (Austral Biologicals, San Ramon, CA), α -tubulin (Merck Calbiochem), and GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and specific proteins were visualized by chemiluminescence.

3. Results

3.1. Assay development

To establish an efficient RT-PCR-based screen for anti-HCV agents, we searched for methods that could be carried out without labor-intensive RNA isolation. We tested whether tube-trapping methods used to obtain plant viral RNAs for RT-PCR (Rowhani et al., 1995; James, 1999; Suehiro et al., 2005) could be applied to HCV. A JFH1 stock solution (3×10^5 ffu/ml, 3×10^7 copies/ml) was serially diluted fourfold, put into the wells of the PCR plate, incubated on ice for 30 min, and then centrifuged at 3500 rpm for 15 min. The supernatant was removed and quantitative RT-PCR was performed by direct addition of the one-step RT-PCR reaction mixture. We found that HCV, like plant viruses, are adsorbed onto the well wall during incubation. As shown in Fig. 1a, RNA adsorption appeared to be linear over a broad range of viral concentrations. HCV RNA could still be detected after seven fourfold dilutions, indicating that the "tube-capture" is a quantitative method that can detect less than 200 copies of HCV RNA. We compared the CT values from this "tube-capture method" with those from the conventional method of RNA extraction. The efficiency of RNA recovery by "tube-capture" was calculated to be about 9% of the conventional method. However, as shown in Fig. 1a, there was a close correlation between the CT values obtained from the two methods ($R = 0.988$), demonstrating the usefulness of this method.

3.2. Identification of BIM I as an inhibitor of HCV infection

To explore the possibility of tube-capture-RT-PCR as a simple screen for discovering anti-HCV compounds, we first tested whether the method would detect the antiviral activity of IFN- α . Huh7.5.1 cells were seeded in a 96-well plate and infected with HCV-JFH1 at an MOI of 0.01. After 5 days, HCV RNA released in the medium was assayed by the tube-capture method. Under these conditions, the CT from the control medium was usually about 18–20. As shown in Fig. 1b, a substantial reduction in the amount of HCV RNA was observed when the cells were infected in the presence of IFN- α .

For further validation of the ability of the assay to identify HCV inhibitors, we performed a pilot screen using an inhibitor kit provided by the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit contains 95 inhibitors including cyclosporin A, a compound reported to inhibit HCV replication.

Cyclosporin A was identified (Fig. 1c), providing a proof of concept for screening for anti-HCV drug candidates. In addition, our assay also identified the PKC inhibitor bisindolylmaleimide I (BIM I) (Fig. 2a, black columns). The IC_{50} was about 0.1 μ M, which is comparable to that of cyclosporin A and about 200-fold lower than the IC_{50} for cell growth (Fig. 2b). In addition, BIM I inhibited the cytopathic effect of HCV JFH1. Infection with HCV resulted in about a 20% reduction of cell growth. BIM I at 1 μ M enhanced the growth of

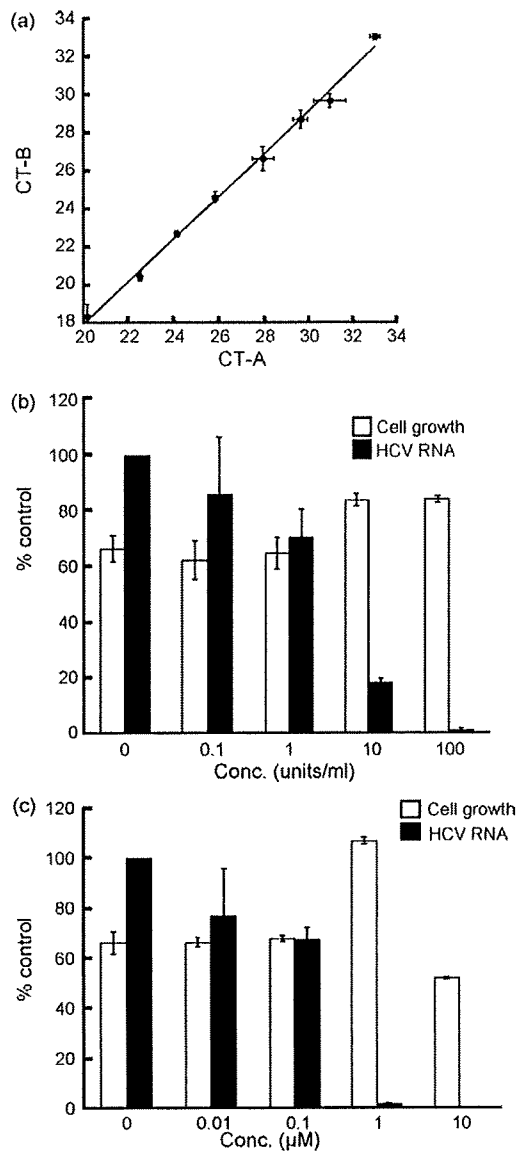


Fig. 1. RT-PCR-based screen for anti-HCV agents using the JFH1 viral culture system. (a) Correlation between CT values from “tube-capture-RT-PCR” (CT-A) and ordinary RNA extraction (CT-B). JFH1 stock solution (3×10^5 ffu/ml) was serially diluted four-fold and quantitative real-time PCR was performed as described under Section 2. CT-A was the average CT of three wells using tube-capture-RT-PCR and CT-B was the average CT of three HCV RNA eluates using a kit (QIAamp Viral RNA mini). (b) and (c) Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of IFN- α (b) or cyclosporin A (c). HCV RNA in the medium (closed (black) columns) was assessed by “tube-capture-RT-PCR” as described under Section 2. Open (white) columns represent percentage of cell growth compared with that of control cells without virus and compound. Columns, mean of triplicate wells; bars, SD.

infected cells, almost to the level of uninfected cells (Fig. 2a, white columns). Recovery of cell growth was also observed with INF- α or cyclosporin A treatment (Fig. 1b and c). BIM I reduced cell growth of uninfected cells only at concentrations of 1 μ M or higher (Fig. 2b). BIM I also inhibited the production of the HCV core protein with marginal effects on host α -tubulin levels (Fig. 2c).

3.3. BIM I inhibits HCV replication

To our knowledge, the anti-HCV effects of BIM I or other PKC inhibitors have not been reported. Because the majority of current

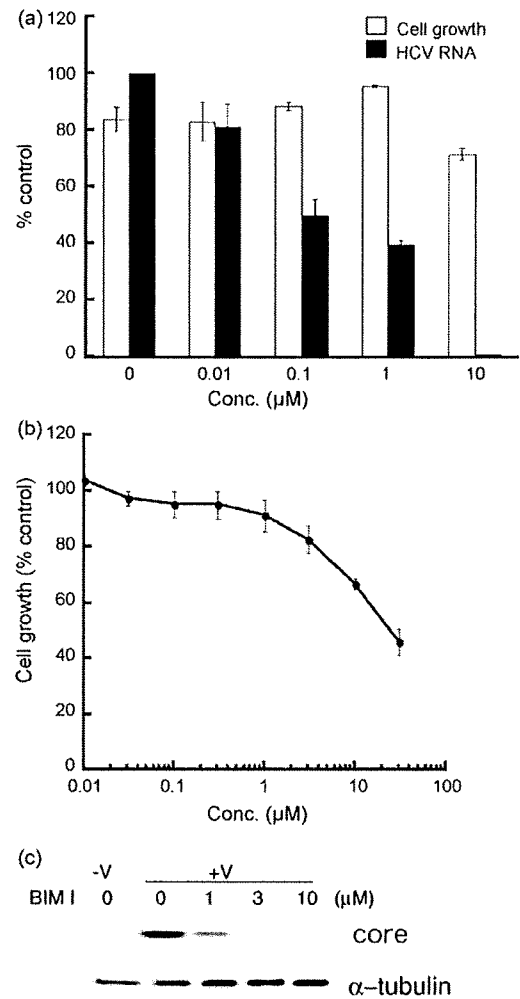


Fig. 2. BIM I inhibits HCV. (a) Effects of BIM I on HCV JFH1 RNA. Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of BIM I and assayed for HCV RNA and cell growth as in Fig. 1. Columns, mean of triplicate wells; bars, SD. (b) Effects of BIM I on growth of Huh 7.5.1 cells. (c) Effects of BIM I on HCV core protein in cells. Cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of BIM I and cultured for 2 days. Cells were lysed and subjected to western blotting as described under Section 2. “V” indicates infection with HCV.

HCV drug screening relies on replicon-based models, we investigated the possibility that BIM I targets a step in the HCV life cycle that is not included in the replicon systems, such as attachment, entry or release. We treated two subgenomic replicon cells with BIM I and examined the amount of NS5A protein.

As shown in Fig. 3a and b, BIM I dose-dependently reduced NS5A in both 1b and 2a subgenomic replicon cells, but not the host GAPDH. The results indicate that BIM I inhibits a process involved in the replication of HCV subgenomic replicons. However, although the NS5A level appeared to be more vulnerable, cell growth was substantially suppressed by BIM I (Fig. 3c). Whereas a significant difference between the IC₅₀ for HCV RNA and cell growth was observed in the HCV cell culture system, the reduction of NS5A in replicon cells overlapped with the effects on cell growth.

To further elucidate the stage of the HCV life cycle affected by BIM I, Huh 7.5.1 cells were inoculated with higher titers of JFH1 (MOI 2) and then treated with 3 μ M BIM I, starting at different time points after infection. JFH1 appeared to complete the life cycle in about 48 h, judging from the expression profiles of viral RNA and proteins in cells (Fig. 4a). When BIM I was added at the time of infection,

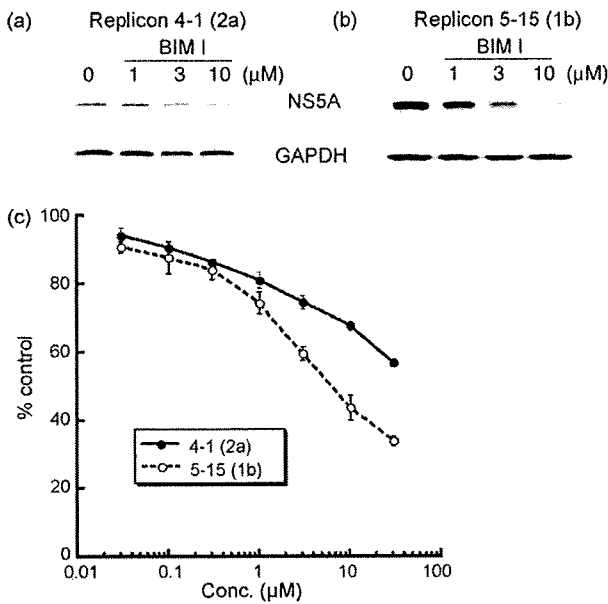


Fig. 3. Effect of BIM I on subgenomic replicon cells. (a) Subgenomic replicon cells harboring genotype 2a (4-1) (a) and genotype 1b (5-15) (b) were treated with BIM I for 2 or 4 days, respectively. Cells were lysed and analyzed by western blotting with anti-NS5A antibody or anti-GAPDH antibody. (c) Effect of BIM I on cell growth of the two replicon cells. Cells were incubated for 2 (4-1) or 4 days (5-15) with BIM I, and cell growth was measured by MTT assay.

the amount of viral RNA in the medium after 48 h decreased to less than 20% of control cells without inhibitor treatment. Addition of BIM I at 6 h post-infection still resulted in a reduction of viral RNA to 30% of control, but after 24 h the antiviral activity of BIM I was significantly diminished and only a modest decline to about 80% was observed (Fig. 4b). These results suggest that interference with RNA synthesis or translation of viral proteins accounts, at least in part, for the anti-HCV activity of BIM I.

3.4. HCV inhibition by bisindolylmaleimide and indolocarbazole compounds does not involve PKC

Since the discovery of staurosporine as a broad-spectrum protein kinase inhibitor, a variety of bisindolylmaleimide and indolocarbazole inhibitors with different potencies and selectivity have been developed. BIM I is one such compound that is highly specific for PKC and is broadly used to analyze PKC-mediated events. To gain insight into the relevance of the PKC inhibitory spectrum and antiviral activity, panels of different bisindolylmaleimide and indolocarbazole compounds were tested in the assays. Contrary to our expectation, no correlation was found between the ability to inhibit PKC and HCV.

Another bisindolylmaleimide PKC inhibitor without the N-dimethylaminopropyl chain, BIM IV, displayed similar significant anti-HCV activity (Fig. 5). Other structurally related pan- and isozyme-specific PKC inhibitors such as BIM II, Ro31-8220 (BIM IX), LY333,531 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (PKCβ inhibitor, Calbiochem) or an indolocarbazole compound K252c also inhibited HCV replication (not shown). However, as shown in Fig. 5, the non-PKC-inhibitory analog BIM V (Toullec et al., 1991) and arcyriaflavin A, an indolocarbazole compound with no reported effects on PKC (Zhu et al., 2003), were also effective in reducing HCV RNA. BIM V was actually more potent than BIM I. Whereas the effect of BIM I on HCV overlapped with cytotoxicity in this particular experiment, BIM V was virtually nontoxic at a dose (1 μM) that reduced

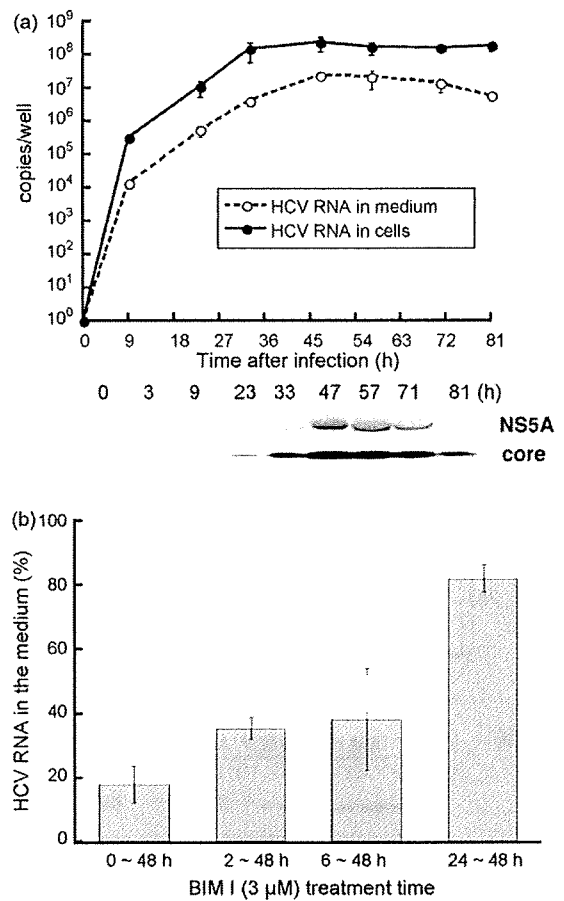


Fig. 4. (a) Expression profile of the HCV RNA and proteins. Huh 7.5.1 cells were infected with HCV as described above, but at an MOI of 2. The cells and medium were harvested at the indicated time and analyzed for HCV RNA and proteins. The core and NS5A protein were detected in the cell lysate. (b) Effect of time of addition. BIM I (3 μM) was added to Huh 7.5.1 cells just before and 2, 6, or 24 h after HCV addition at an MOI of 0.2. After 48 h of incubation following virus infection, the HCV RNA in the medium was extracted and measured with quantitative real-time RT-PCR. The indicated values represent the averages for two independent experiments.

HCV RNA to less than 20% of control. The results indicate that a broad range of bisindolylmaleimide and indolocarbazole compounds inhibit HCV replication, albeit in a PKC-independent manner.

4. Discussion

HCV replicon systems have made significant contributions in HCV research and drug development. Nevertheless, as drug screening tools, replicon systems have limitations because they are not capable of identifying inhibitors of several important events in the viral life cycle. The use of HCV cell culture systems should overcome the drawbacks of the replicon systems and facilitate the identification of inhibitors with novel mechanisms of action. Actually, it has recently been shown that use of an infectious HCV system identified inhibitors that a replicon-based screen did not recognize (Zhang et al., 2008).

We developed a simple screening method for HCV inhibitors that measures the viral RNA released from JFH1-infected cells. The assay does not require specially engineered viruses. The “tube-capture-RT-PCR” method obviates the need for labor-intensive RNA isolation and significantly increases the efficiency of screening. The validity of the assay was confirmed by successful identification of known

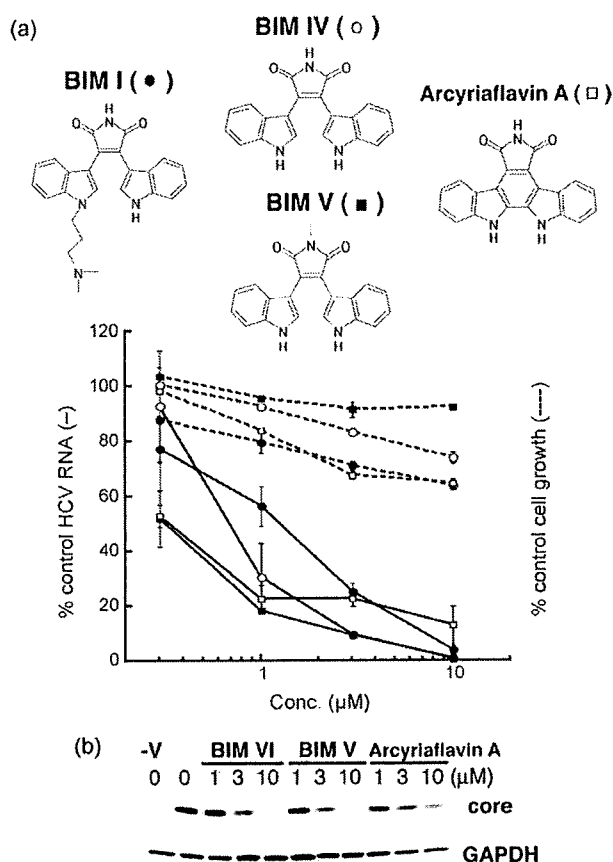


Fig. 5. Effects of bisindolylmaleimide or indolocarbazole compounds on HCV infection. (a) Effects of BIM I, BIM IV, BIM V, and Arcyriaflavin A on HCV RNA in the medium. Huh 7.5.1 cells were seeded and infected with HCV at an MOI of 0.01 in the presence of drugs. After 4 days of incubation, the HCV RNA in the medium was extracted and quantified. The relative amounts of HCV RNA with BIM I (closed circle), BIM IV (open circle), BIM V (closed rectangle), and Arcyriaflavin A (open rectangle) are represented by solid lines. Cell viability, represented by dotted lines, was determined by MTT assay of a parallel culture without HCV challenge. (b) Effects of BIM I, BIM V and Arcyriaflavin A on core proteins in cells. The cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of compounds and analyzed as described in Fig. 2c. “-V” indicates control without HCV infection.

HCV inhibitors in the pilot screen. In addition, the assay identified the PKC inhibitor BIM I.

BIM I is a widely used compound, and it was somewhat surprising to us that its anti-HCV activity had not been reported. HCV replication in the cell culture system appeared to be considerably more susceptible to BIM I than in the replicon systems, and this is probably why this compound had not been identified as an HCV inhibitor.

Because virus replication is closely linked to host cell growth, HCV inhibition could occur as a result of cell growth inhibition. However, as shown in Fig. 2a, BIM I reduced the cytopathic effect of HCV infection just like interferon- α and cyclosporin A. BIM I, at 1 μ M, enhanced the growth of infected cells almost to the level of uninfected cells, presumably because HCV replication, but not cell growth, was inhibited, resulting in a reversal of the cytopathic effect.

Because the PKC inhibitory properties of bisindolylmaleimides and indolocarbazoles have been characterized extensively, we tested a panel of commercially available compounds in the assay to gain insight into the role of PKC in HCV replication. However, no correlation between PKC inhibition and antiviral activity could be found. Anti-HCV activity did not involve PKC inhibition,

apparently because a non-PKC-inhibitory analog, BIM V, was also active. Furthermore, PKC inhibitors with different structures, such as calphostin C and H-7, did not show specific inhibition of HCV (data not shown).

Previous studies have indicated that bisindolylmaleimide PKC inhibitors have cellular targets other than PKC. It has been shown that BIM I and Ro31-8220 inhibit p70S6K and p90RSK (Alessi, 1997; Roberts et al., 2005) and that BIM V inhibits p70S6K (Marmy-Conus et al., 2002). Although we did not monitor the activities of these enzymes in our experiments, inhibition of p70S6K is unlikely to be responsible for the anti-HCV effect of PKC inhibitors, because Ishida et al. reported that silencing of p70S6K enhanced HCV RNA abundance (Ishida et al., 2007).

Bisindolylmaleimides and indolocarbazoles have also been reported to inhibit the ATP-binding cassette (ABC) transporters P-glycoprotein and multidrug resistance-associated protein 1 (MRP1), efflux pumps that play important roles in cancer drug resistance (Merritt et al., 1999; Gekeler et al., 1995). More recently, Robey et al. reported that BIMs I, II, III, IV, and V, K252c, and Arcyriaflavin A inhibit ABCG2, an ABC half-transporter that confers resistance to various antitumor agents (Robey et al., 2007). Whether ABC transporters play any role in HCV infection awaits further study. We are currently examining the anti-HCV effects of other ABC transporter inhibitors.

In conclusion, we developed a simple infectious HCV system-based assay that can be used for high-throughput screening of HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles. These compounds might represent lead substances for the development of new HCV drugs. Further analysis of the mechanism of HCV-inhibition by these compounds might reveal a new mechanism of regulation of HCV infection.

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Tacrolimus Ameliorates Metabolic Disturbance and Oxidative Stress Caused by Hepatitis C Virus Core Protein

Analysis Using Mouse Model and Cultured Cells

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Hepatic steatosis and insulin resistance are factors that aggravate the progression of liver disease caused by hepatitis C virus (HCV) infection. In the pathogenesis of liver disease and metabolic disorders in HCV infection, oxidative stress due to mitochondrial respiratory chain dysfunction plays a pivotal role. Tacrolimus (FK506) is supposed to protect mitochondrial respiratory function. We studied whether tacrolimus affects the development of HCV-associated liver disease using HCV core gene transgenic mice, which develop hepatic steatosis, insulin resistance, and hepatocellular carcinoma. Administration of tacrolimus to HCV core gene transgenic mice three times per week for 3 months led to a significant reduction in the amounts of lipid in the liver as well as in serum insulin. Tacrolimus treatment also ameliorated oxidative stress and DNA damage in the liver of the core gene transgenic mice. Tacrolimus administration reproduced these effects in a dose-dependent manner in HepG2 cells expressing the core protein. The intrahepatic level of tumor necrosis factor- α , which may be a key molecule for the pathogenesis in HCV infection, was significantly decreased in tacrolimus-treated core gene transgenic mice. Tacrolimus thus reversed the effect of the core protein in the patho-

genesis of HCV-associated liver disease. These results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis. (Am J Pathol 2009, 175:1515-1524; DOI: 10.2353/ajpath.2009.090102)

Hepatitis C virus (HCV) is a major cause of liver disease; approximately 170 million people are chronically infected worldwide. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem from both medical and socioeconomic viewpoints.^{1,2} Recently, a growing amount of evidence showing that HCV infection induces alteration in lipid³⁻⁷ and glucose metabolism has accumulated.^{8,9} Augmentation of oxidative stress is also substantiated in HCV infection by a number of clinical and basic studies.¹⁰⁻¹³

We demonstrated previously that the core protein of HCV induces HCC in transgenic mice that have marked hepatic steatosis in the absence of inflammation.¹⁴ In this animal model for HCV-associated HCC, there is augmentation of oxidative stress in the liver during the incubation period.¹⁰ Also noted is an accumulation of lipid droplets that are rich with carbon 18 monounsaturated fatty acids such as oleic and vaccenic acids, which is also observed in liver tissues of patients with chronic hepatitis C com-

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pared with those in patients with fatty liver due to simple obesity.¹⁵ Recently, we have also shown, using the HCV transgenic mouse model, that the ability of insulin to lower plasma glucose levels is impaired in association with HCV infection,¹⁶ which would be the basis for the frequent development of type 2 diabetes in patients with chronic hepatitis C.^{8,9}

Disturbances in lipid and glucose metabolism are notable features of HCV infection and may be profoundly involved in the pathogenesis of liver diseases. Although the mechanism underlying these phenomena is not yet well understood, the development of clues to correct these metabolic disturbances occurring in HCV infection, which have been recently connected to the poor prognosis of patients with chronic hepatitis C, is awaited. Moreover, a key role for oxidative stress in the pathogenesis of hepatitis C,^{11,12} which may be closely associated with the aforementioned metabolic disorders, has been identified. The association of oxidative stress augmentation in HCV infection with mitochondrial respiratory dysfunction^{10,13,17} suggests that one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function.

We have conducted information retrieval and screening for agents that can protect the mitochondrial respiratory function. Tacrolimus (FK506), which is widely used in organ transplantation, is one such agent with evidence showing protection of the mitochondrial respiratory function,¹⁸⁻²¹ although it shows no antiviral effect. We explored, using transgenic mouse and cultured cell models that express the HCV core protein, whether tacrolimus improves metabolic disturbances including lipid and glucose homeostases as well as oxidative stress augmentation through a possible involvement of mitochondrial function.

Materials and Methods

Transgenic Mouse and Cultured Cells

The production of HCV core gene transgenic mice has been described previously.⁶ Mice were cared for according to institutional guidelines with the approval by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd., Tokyo, Japan), and maintained in a specific pathogen-free state. Because there is a sex preference in the development of liver lesions in the transgenic mice, we used only male mice. At least five mice were used in each experiment, and the data were subjected to statistical analysis. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously.^{22,23} Bulk HepG2 cells were also used as a control.

Reagents

Cholesterol esters and lipid standards were purchased from Sigma-Aldrich (St. Louis, MO), and glycogen and

amyloglucosidase were obtained from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were of analytical grade and were purchased from Wako Chemicals (Tokyo, Japan). Tacrolimus (FK506) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Cyclosporine A (CyA) was purchased from Sigma-Aldrich.

Administration of Tacrolimus and Cyclosporine A

Tacrolimus (0.1 mg/kg b.wt., suspended in mannitol and hydroxychlorinated castor oil [HCO-60]), or vehicle only was administered to the core gene transgenic or control mice i.p., three times per week for 3 months beginning at 3 months of age. For *in vitro* experiments, tacrolimus was added to the culture medium at the final concentration of 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. CyA was also added to the culture medium at the same concentrations.

Assessment of Glucose Homeostasis

Blood was drawn at different time points from the tail vein, and plasma glucose concentrations were measured using an automatic biochemical analyzer (DRI-CHEM 3000V, Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (Biotrak, Amersham Pharmacia Biotech, Piscataway, NJ) using rat insulin as a standard. For the determination of the fasting plasma glucose level, the mice were fasted for >16 hours before the study. An insulin tolerance test was performed as described previously.¹⁶

Lipid Extraction, Measurement of Triglyceride Content, and Analysis of Fatty Acid Compositions

Lipid extraction from the mouse liver tissues or cultured cells was performed as described previously.^{15,24} For the analysis of fatty acid compositions, the residue was methylated by the modified Morrison and Smith method with boron trifluoride as a catalyst.²⁵ Fatty acid methyl esters were analyzed using a Shimadzu GC-7A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 30-m-long \times 0.3-mm diameter support coated with ethylene glycol succinate.²⁴

Evaluation of Oxidative and Antioxidative System

Lipid peroxidation was estimated spectrophotometrically using thiobarbituric acid-reactive substances and is expressed in terms of malondialdehyde formed per milligram protein. Reduced glutathione and oxidized glutathione levels were measured as described previously.¹⁰ The total amount of glutathione was calculated by adding the amounts obtained for glutathione and oxidized glutathione. For the evaluation of DNA damage in cells, apurinic/aprimidinic sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technolo-

gies, Inc., Tokyo, Japan) following the manufacturer's protocol.

Determination of Reactive Oxygen Species

Cells were plated onto glass coverslips and examined for reactive oxygen species (ROS) production as a marker for oxidative stress. They were loaded for 2 hours with chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes Inc., Eugene, OR) at a final concentration of 10 $\mu\text{mol/L}$.²⁶ Results were expressed as relative fluorescence intensity and normalized to the control cells. In some experiments, ROS was measured after the incubation with tacrolimus or CyA.

Measurement of Ketone Body Ratio

For the determination of ketone body ratio (KBR), cells were cultured to confluence on a 3.5-cm dish, and the medium was replaced with 700 μl of fresh medium. For arterial KBR, the mice were fasted for >16 hours, followed by the drawing of arterial blood. After a 24-hour incubation, acetoacetate and β -hydroxybutyrate in the medium were measured by monitoring the production or consumption of NADH with a Ketorex kit (Sanwa Chemical, Nagoya, Japan).²⁷ The KBR was calculated as the acetoacetate/ β -hydroxybutyrate ratio.

Microarray Analysis

An Affymetrix GeneChip analysis cDNA array system (Mouse Genome 430A 2.0, Kurabo, Osaka, Japan) was used for the analysis. Two thousand species of mouse DNA fragments were spotted on the filter. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with mice treated with vehicle were defined as up-regulated or down-regulated, respectively.

Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescent signal was measured with an ABI Prism 7000 system (Applied Biosystems, Tokyo, Japan).

The genes encoding mouse *tumor necrosis factor (TNF)- α* , *sterol regulatory element binding protein (SREBP)-1c*, *resistin*, *stearoyl-CoA desaturase (SCD)-1*, and *hypoxanthine phosphoribosyltransferase* were amplified with the primer pairs 5'-GACAAGGTGGGCTACGGGCTTG-3' and 5'-TCCCAAATGGGCTCCCTCT-3', 5'-ACGGAGCCATGG-ATTGCACATTTG-3' and 5'-TACATCTTTAAAGCAGCGG-GTGCCGATGGT-3', 5'-GAAGGCACAGCAGTCTTGA-3' and 5'-GCGACCTGCAGCTTACAG-3', 5'-TTCCCTCTG-CAAGCTCTAC-3' and 5'-CGCAAGAAGGTGCTAAC-GAAC-3', and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' and 5'-GTAATGATCAGTCAACGGGGGAC-3', respec-

tively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

Reporter Assay for SREBP-1c Promoter Activity

A plasmid encoding firefly luciferase under the control of the *SREBP-1c* promoter (pGL3-srebp-1cPro) and a control plasmid encoding *Renilla* luciferase (Promega, Madison, WI) were transfected into 293T cells. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium of 293T cells transfected with pGL3-srebp-1cPro with or without an expression plasmid of HCV core protein at 24 hours after transfection. Cells were harvested 24 hours after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold-increase in relative luciferase units.

Statistical Analysis

Data are presented as the mean \pm SE. The significance of the difference in means was determined by a Mann-Whitney *U* test wherever appropriate. $P < 0.05$ was considered significant.

Results

Effect of Tacrolimus on Insulin Resistance Induced by HCV

The *core gene* transgenic mice exhibit insulin resistance in the absence of obesity from the age of 2 months.¹⁶ In tacrolimus-treated mice, there was a slight, but not significant, reduction in body weight compared with control mice at the end of tacrolimus administration at 6 months of age (Figure 1A). Tacrolimus administration to the *core gene* transgenic mice restored the plasma glucose levels to within normal limit (Figure 1B) ($P < 0.05$), whereas it caused no significant reduction in the control mice. The plasma glucose levels in the vehicle-treated *core gene* transgenic mice were higher than those in the *core gene* transgenic mice reported previously,¹⁶ probably owing to the older age of mice in the current study than in the previous one. The levels of serum insulin were also significantly reduced by the treatment with tacrolimus for 3 months in the *core gene* transgenic mice, whereas there was no significant change in the control mice (Figure 1C). The reduction in both plasma glucose and serum insulin levels indicates that the administration of tacrolimus restored the resistance to insulin action, which is attributed to the suppression of insulin action in the liver by the *core protein*.¹⁶ Actually, an insulin tolerance test (1 U/kg b.wt.) demonstrated the improvement of insulin action in the tacrolimus-treated *core gene* transgenic mice (Figure 1D).

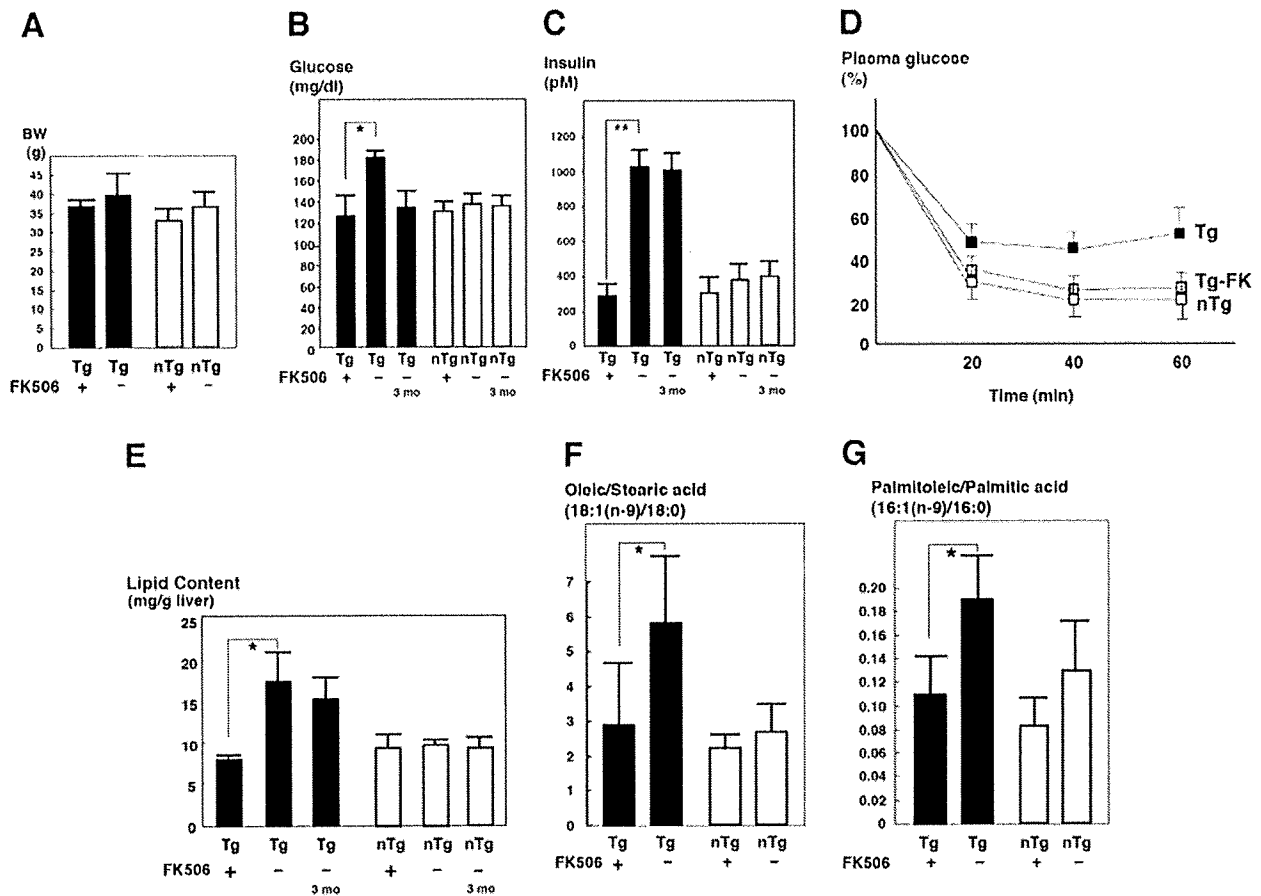


Figure 1. Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline and end of treatment. **B:** Plasma glucose level. **C:** Serum insulin level. **D:** Insulin tolerance test. Black boxes represent core gene transgenic mice; white boxes represent control mice; gray boxes represent core gene transgenic mice treated with tacrolimus (Tg-FK). **E:** Total lipid content in the liver. **F:** Ratio of oleic/stearic acid [18:1(n-9)/18:0]. **G:** Ratio of palmitoleic/palmitic acid [16:1(n-9)/16:0]. black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice showing the baseline state just before FK treatment, and Tg indicates 6-month-old transgenic mice, either with or without tacrolimus treatment for 3 months. Values represent the mean \pm SE, $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. ** $P < 0.01$.

Tacrolimus Improves Lipid Metabolism Disorders in Mice

We then studied whether tacrolimus administration affects lipid metabolism in the mice. The core gene transgenic mice developed a marked hepatic steatosis.^{6,14} In addition, the composition of accumulated lipid was different from that in the fatty liver as a result of simple overnutrition: carbon 18 or 16 monounsaturated fatty acid levels were significantly increased.¹⁵ As shown in Figure 1E, the tacrolimus treatment significantly reduced the lipid content in liver tissues compared with the vehicle treatment of the core gene transgenic mice ($P < 0.05$, $n = 5$ each), whereas there was no change in the control mice. The increased ratios of oleic to stearic acid [18:1(n-9)/18:0] and palmitoleic to palmitic acid [16:1(n-9)/16:0] in the core gene transgenic mice returned to levels similar to those in control mice (Figure 1, F and G) ($P < 0.05$). Thus, the administration of tacrolimus for 3 months restored the abnormalities in lipid metabolism that were induced by the core protein of HCV. Histologically, tacrolimus significantly improved steatosis in the liver of

core gene transgenic mice, in which micro- and macrovesicular lipid droplets were accumulated in hepatocytes, chiefly around the central veins of the liver (Figure 2A). There was no sign of inflammation in the liver with or without the tacrolimus treatment.

Effect of Tacrolimus on Lipid Metabolism in HepG2 Cells Expressing HCV Core Protein

To further prove the ameliorating effect of tacrolimus on lipid metabolism, we then performed experiments using HepG2 cells that express the core protein.^{22,23} HepG2 cells, the lipid metabolism of which is somewhat different from that in normal hepatocytes,²⁸ show a significant increase in the level of 5,8,11-eicosatrienoic acid [20:3(n-9)], as a result of activations of the fatty acid enzymes, Δ^9 -, Δ^6 -, and Δ^5 -desaturases, by the core protein (H. Miyoshi and K. Koike, unpublished data). Incubation of the core-expressing HepG2 cells with tacrolimus at 100 nmol/L and 1 μ mol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

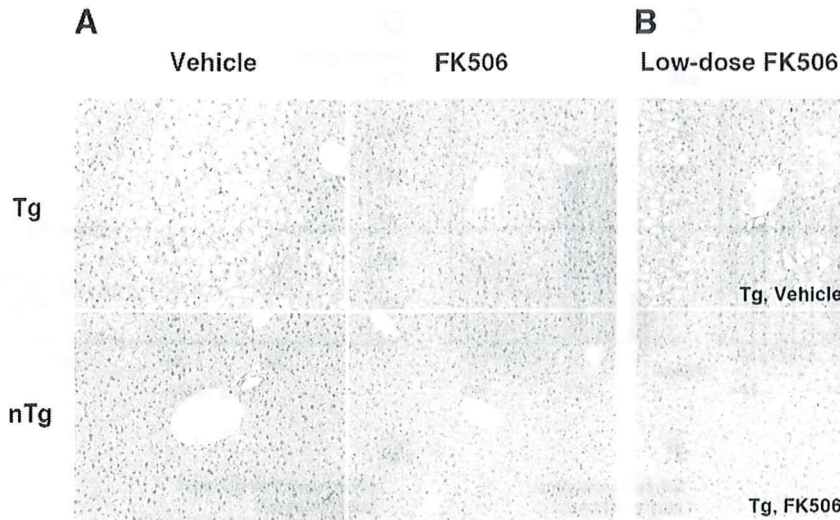


Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). **A:** There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with the vehicle-treated mice. **B:** A prominent improvement in steatosis was also obtained by the administration of one-fifth dose of tacrolimus for 1 month beginning at 3 months of age. For histological analysis, two independent researchers evaluated 40 microscopic fields each, and a representative picture is shown for each category. Original magnification, $\times 125$. Tg, transgenic mice; nTg, nontransgenic control mice.

ment increased the level of 20:3(n-9) in a dose-dependent manner in the core-expressing HepG2 cells (Figure 3, A and B). Neither tacrolimus nor CyA changed the 20:3(n-9) content in HepG2 cells that do not express the core protein.

Low Dose of Tacrolimus Also Ameliorates Steatosis and Insulin Resistance

Because the usual dose of tacrolimus for liver transplantation naturally induces an immunosuppressed state in patients, we conducted a mouse study with a tacrolimus dose lower than that in the aforementioned study. In this low-dose experiment, tacrolimus at 0.02 mg/kg b.wt. (one-fifth of the previous one) was administered to mice for 1 month from the age of 3 months. Similar to the results with the dose of 0.1 mg/kg b.wt., there were significant decreases in the lipid content in the liver (9.5 ± 0.8 [0.02 mg/kg b.wt. tacrolimus] versus 18.7 ± 4.4 [vehicle only] mg/g liver; $P < 0.05$) and serum insulin concentration (96.6 ± 16.9 [0.02 mg/kg b.wt. tacrolimus] versus 1137.1 ± 88.0 [vehicle only] pmol/L; $P < 0.05$) in

the core gene transgenic mice treated with tacrolimus. Histological changes are shown in Figure 2B.

Effect of Tacrolimus on Oxidative Stress and Antioxidative System in Mice

We next examined whether the 3-month administration of tacrolimus affects the redox state in the core gene transgenic mice. In the liver of the core gene transgenic mice, the ROS level was higher than that in the liver of control mice as determined by lipid peroxidation.¹⁰ Treatment with tacrolimus significantly reduced the level of thiobarbituric acid-reactive substances in the liver of the core gene transgenic mice (Figure 4A) ($P < 0.05$). As a result of oxidative stress overproduction, there was damage in the DNA of hepatocytes of the core gene transgenic mice from a young age.¹⁰ To evaluate the effect of tacrolimus on the nuclear DNA damage, the apurinic/aprimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/aprimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

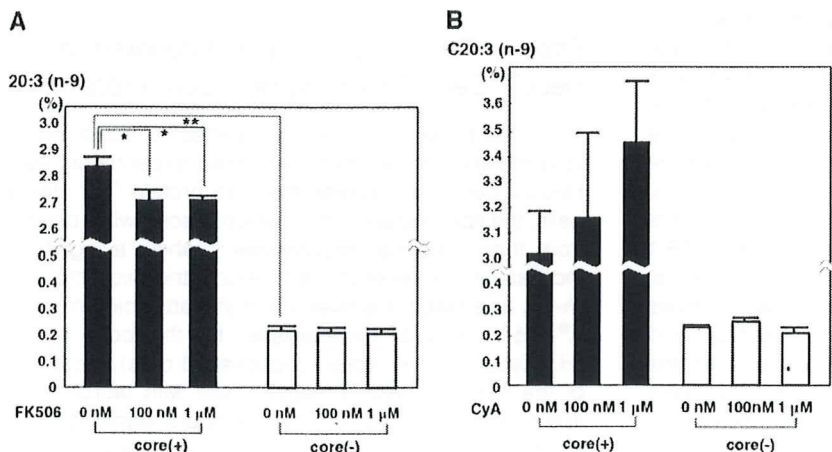


Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. **A:** Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μ M. **B:** Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μ M. Black bars represent core-expressing cells; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$ and ** $P < 0.01$.

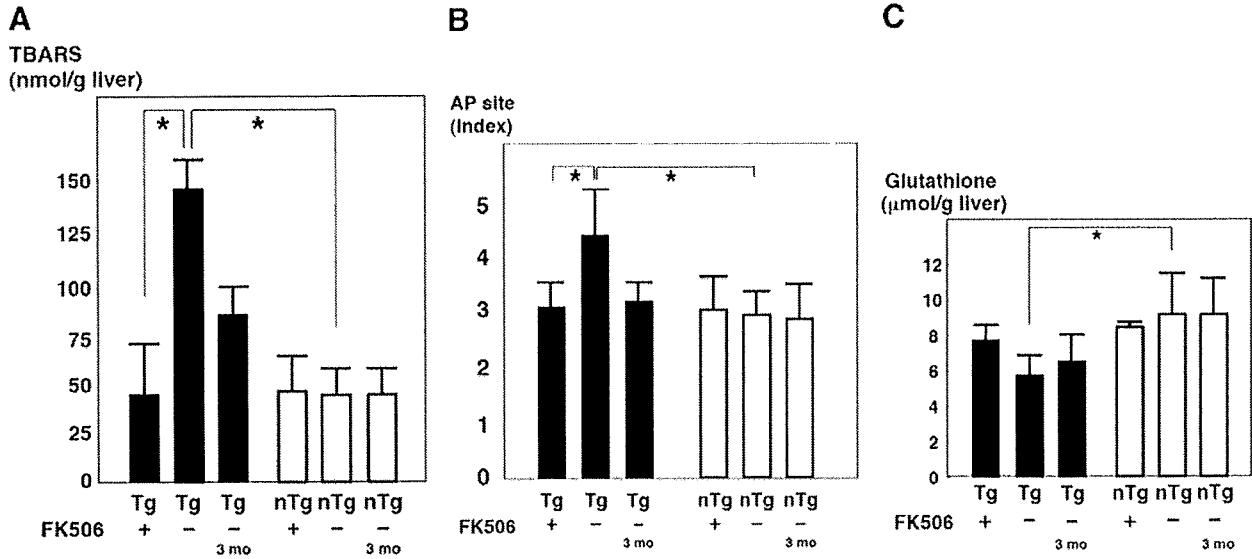


Figure 4. Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/aprimidinic (AP) site in the liver as a marker of nuclear DNA damage; **C:** Total glutathione level in the liver. Black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice, showing the baseline state just before tacrolimus treatment, and Tg indicates 6-month-old transgenic mice, either with or without 3 months of tacrolimus treatment. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. TBARS, thiobarbituric acid-reactive substances.

than that in the control mice, was significantly decreased by the tacrolimus treatment to a level similar to that in the control mice ($P < 0.05$).

The level of glutathione, one of the antioxidant systems, was significantly decreased in the liver of the core gene transgenic mice presumably as a result of oxidative stress overproduction but returned to a level similar to that in the control mice after the 3-month administration of tacrolimus, although the difference was not statistically significant ($P = 0.063$) (Figure 4C). Thus, the oxidative stress augmentation induced by the core protein of HCV was reduced by tacrolimus.

Effect of Tacrolimus on Oxidative Stress in Core-Expressing HepG2 Cells

Evidence for scavenging ROS by the administration of tacrolimus to the mice prompted us to validate this finding using cultured cells. For this purpose, tacrolimus or CyA was added to the culture medium of HepG2 cells that express or do not express the core protein. After 24 hours of incubation, tacrolimus decreased the ROS production level in the core-expressing HepG2 cells in a dose-dependent manner (Figure 5A). In contrast, no decrease but rather an augmentation of ROS production was observed by the treatment with CyA at various concentrations (Figure 5B).

Because dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be the reason for the ROS production associated with HCV infection (H. Miyoshi and K. Koike, unpublished data),^{12,13,17} an increase in the NADH/NAD⁺ ratio, which is caused by the repression of the complex 1 NADH dehydrogenase activity, would be a good marker for the mitochondrial complex 1 dys-

function. Therefore, we evaluated the effect of tacrolimus on the accumulation of NADH in the core-expressing HepG2 cells. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR,^{26,29} was significantly higher in the core gene transgenic mice than in control mice (1/atrial KBR) and in HepG2 cells expressing the core protein than in control cells (1/KBR) (Figure 6A). By the treatment with 1 μ mol/L tacrolimus, the ratio significantly decreased compared with the baseline (Figure 6B), whereas CyA treatment caused no effect in the core-expressing HepG2 cells (Figure 6C), as was the

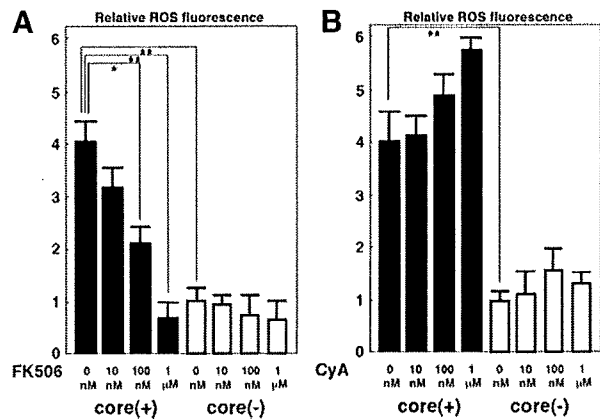


Figure 5. Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$; ** $P < 0.01$.

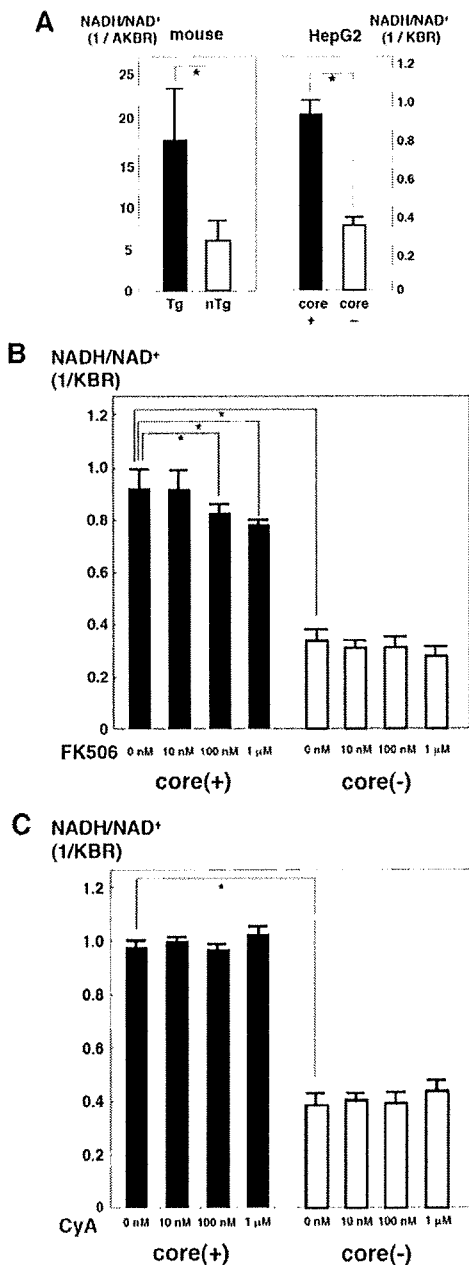
case in the determination of ROS by chloromethyl 2',7'-dichlorodihydrofluorescein diacetate.

Changes in Gene Expression by Tacrolimus Treatment of Mice

We then performed a comprehensive microarray analysis of gene expression in the liver, which was up- or down-regulated by tacrolimus. For this analysis, the tacrolimus-treated mice were compared with the vehicle-treated mice, in two pairs of the core gene transgenic and control mice, respectively. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice

compared with those treated with vehicle were defined as up-regulated or down-regulated, respectively. As shown in Table 1, several genes were found to be up-regulated or down-regulated in both the core gene transgenic and control mice after the treatment with tacrolimus for 3 months. A number of genes including that for TNF- α were up- or down-regulated both in the core gene transgenic and control mice. In contrast, the expressions of some genes including that for resistin were differentially regulated between the core gene transgenic and control mice. The expressions of these genes were confirmed by real-time PCR analysis.

Then, to explore the mechanism by which tacrolimus reverses the pathological effect of the core protein in the liver, we examined, by real-time PCR analysis, the expression of some cellular genes including TNF- α , SREBP-1c, SCD-1, and proteasome activator 28- γ . These genes or gene products have been suggested to play a pivotal role in the pathogenesis of HCV-associated liver disease.^{30,31} TNF- α and SREBP-1c genes have been shown to be up-regulated in the liver of the core gene transgenic mice and considered to play a role in the development of insulin resistance and steatosis.^{30,31} By the treatment of the core gene transgenic mice with tacrolimus for 3 months, there was a significant decrease in the mRNA level of both TNF- α and SREBP-1c (Figure 7, A-C) ($P < 0.05$). The SCD-1 mRNA level was also reduced in the tacrolimus-treated core gene transgenic mice. Because down-regulation of SREBP-1c expression by tacrolimus was observed only in the core gene transgenic mice but not in control mice, it is estimated that tacrolimus antagonizes the action of core protein in its transactivating function of the SREBP-1c promoter. The down-regulation of SREBP-1c, then, would lead to the suppression of SCD-1 expression and amelioration of steatosis. We confirmed this by conducting luciferase assays using cultured cells. As shown in Figure 7D, tacrolimus cancelled the effect of the core protein on the activation of SREBP-1c gene promoter. The level of the proteasome activator 28- γ protein, which is indispensable for the action of the core protein in the pathogenesis of HCV-associated liver lesion,³¹ was determined by Western blotting, but there was no change caused by the tacrolimus treatment (data not shown).



Discussion

Antiviral treatment for chronic hepatitis C has advanced markedly. Nearly 50% of patients with chronic hepatitis C

Figure 6. Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD⁺ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **C:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with CyA for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396 and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

Table 1. Genes Whose Expression Levels in the Mouse Liver Were Altered by the Treatment with FK506

	Up-regulated in Tg	Down-regulated in Tg
Up-regulated in nTg	Nuclear factor, erythroid derived 2 DNA segment, human D6S2654E Fatty acid binding protein 5 epidermal squalene epoxidase	Resistin Resistin like alpha Nuclear receptor subfamily 4, group A, member insulin-like growth factor binding protein 1 calcium and integrin binding family member 3
Down-regulated in nTg	Zinc finger protein 69 X-linked lymphocyte-regulated 4 Cytochrome P450, family 2, subfamily b, polypeptide 9 X-linked lymphocyte-regulated 3a Signal sequence receptor, delta	Tumor necrosis factor alpha Cytochrome P450, family 17, subfamily a, polypeptide 1 B-cell leukemia/lymphoma 6

Genes with altered expression in Tg (columns) or in nTg (rows) are described in a 4 × 4 table. Genes that were 1.5-fold increased or decreased in both of the two FK506-treated mice compared with those treated with placebo were defined as up-regulated or down-regulated, respectively. Tg, core gene transgenic mouse; nTg, nontransgenic control mouse.

with HCV genotype 1 and high viral loads achieve a sustained virological response as a result of ribavirin/peginterferon combination therapy.^{32,33} However, the remaining patients who could not achieve sustained virological response continue to experience progression of chronic hepatitis and have a high probability for development of HCC. Although therapies with new agents such as viral protease or RNA polymerase inhibitors are being developed, there is hope for development of the means to retard the progression of chronic hepatitis.

Recently, evidence showing that hepatic steatosis and insulin resistance are crucial determinants of the progression of liver fibrosis has accumulated.³⁴⁻³⁷ Moreover, the importance of oxidative stress, which is closely associated with metabolic disorders such as insulin resistance and steatosis, is implicated in the pathogenesis of HCV-associated liver disease. Given the suggested association of oxidative stress augmentation with the dysfunction of mitochondrial respiration in HCV infection,^{12,13,17} one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function. Tacrolimus is one such agent with evidence of providing protection of the mitochondrial respiratory function,¹⁸⁻²¹ although it does not show an antiviral effect.

In the current study, the administration of tacrolimus significantly improved the disturbances in lipid and glucose metabolism both *in vivo* and *in vitro*. As disorders of

lipid metabolism associated with HCV infection, hepatic steatosis and increases in monounsaturated fatty acid levels have been demonstrated.^{3,4,6,7,15} The latter is caused by the activation of fatty acid enzymes such as Δ^9 - or Δ^6 -desaturase, resulting in increases in 18:1(n-9)/18:0 and 16:1(n-9)/16:0 ratios (H. Miyoshi and K. Koike, unpublished data).¹⁵ Tacrolimus ameliorated these lipid alterations associated with HCV infection with no impact on mouse body weight. Tacrolimus also improved the insulin resistance in the HCV mouse model, in which tyrosine phosphorylation of insulin receptor substrate-1 is impaired by the HCV core protein.¹⁶

Moreover, tacrolimus treatment ameliorated oxidative stress augmentation, which is considered to play a pivotal role in the progression of liver disease or the development of HCC in HCV infection.¹⁰⁻¹³ In mice transgenic for the HCV core gene, in which DNA damage develops because of oxidative stress augmentation,¹³ tacrolimus decreased the levels of peroxy lipid and DNA damage formations. Dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be a source of ROS overproduction in HCV infection.^{12,13,17} To assess changes in mitochondrial complex 1 function caused by tacrolimus, the NADH/NAD⁺ ratio, which reflects the complex 1 NADH dehydrogenase activity, was determined in HepG2 cells expressing the core protein. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR (1/atrial KBR),^{26,29} was significantly re-

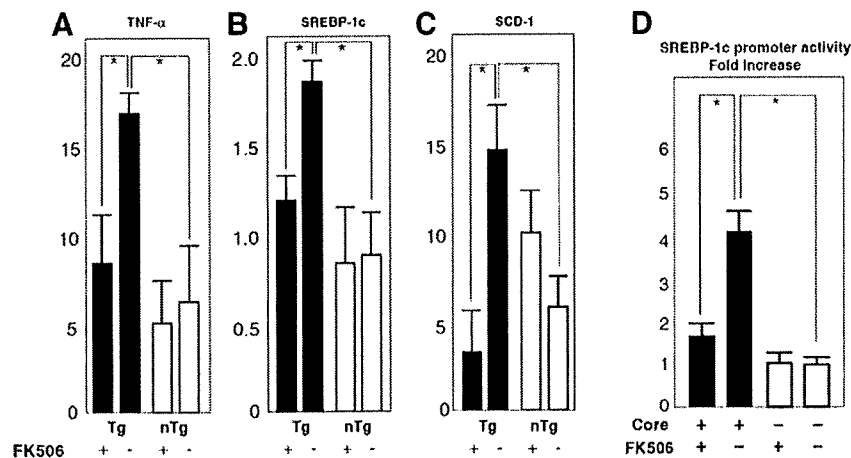


Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF- α (A), SREBP-1c (B), and SCD-1 (C) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. D: Effect of tacrolimus on the transactivating function of the core protein on the SREBP-1c promoter. A luciferase assay was performed using a plasmid encoding firefly luciferase under the control of the SREBP-1c promoter with or without the expression of HCV core protein. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium. Black bars represent transgenic mice; white bars represent control cells. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic mice.