Expression of Gastrointestinal Glutathione Peroxidase Is Inversely Correlated to the Presence of Hepatitis C Virus Subgenomic RNA in Human Liver Cells*

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There is great medical need to develop novel therapies for treatment of human hepatitis C virus (HCV). By gene expression analysis of three HCV-subgenomic RNA replicon cell lines, we identified cellular proteins whose expression is affected by the presence of HCV and therefore may serve as drug targets. Data from cDNA array filter hybridization, as well as from Northern and Western blotting, revealed that the gastrointestinal-glutathione peroxidase (GI-GPx) was drastically down-regulated (up to 20-fold) in all replicon cell lines tested. Concomitantly, total cellular glutathione peroxidase activity was drastically reduced, which rendered these human liver cells more susceptible toward oxidative stress. Interferon α caused down-regulation of the HCV-replicon followed by recovery of GI-GPx expression to nearly normal levels. Furthermore, expression of GI-GPx in replicon cells by gene transduction caused down-regulation of HCV RNA in a dose-dependent manner. Moreover, activating the endogenous gene coding for GI-GPx by all-trans-retinoic acid (RA) was sufficient to cause down-regulation of the HCV replicon. A small interfering RNA duplex abrogated GI-GPx up-regulation by RA and concomitantly suppression of HCV. The RA effect was dependent on the presence of sodium selenite, was reversible, and was independent of RNA-activated protein kinase. Taken together, these results show that HCV inhibits the expression of GI-GPx in replicon cells to promote its intracellular propagation. Modulation of GI-GPx activity may open new avenues of treatment for HCV patients.

The hepatitis C virus (HCV) infects ~2% of the population worldwide (1), and more than 80% of these patients become chronically infected causing recurring hepatitis, liver cirrhosis, and cancer. HCV-related end-stage liver disease is now the leading reason for liver transplantation. Interferon (IFN) monotherapy is effective in only 10% of HCV patients (2–4), and the combination of pegylated interferon and ribavirin can eradicate the virus in about 50% of patients (reviewed in Ref. 5). The mechanism of action of ribavirin has not been fully determined, but incorporation of ribavirin triphosphate into the HCV genome seems to induce error-prone replication to a nontolerable mutation rate (6). Binding of IFNα/β leads to the dimerization of the respective receptors and activation of the Jak/STAT pathway, which turns on cellular genes known to be involved in antiviral responses (7). Among these are the Mx proteins, major histocompatibility antigens, 2',5'-oligoadenylate synthase, and the double-stranded RNA-specific protein kinase (PKR). The precise mechanisms by which HCV can resist the IFN response is not fully understood, but viral proteins E2 and NS5A seem to play a role in evasion of the cellular defense system by blocking PKR (reviewed in Ref. 8).

Molecular studies on HCV-host cell interaction have been hampered by the lack of small animal model systems (reviewed in Ref. 9). To overcome these limitations, a human hepatoma cell line (HuH7) was transfected with subgenomic HCV RNAs, called replicons (Fig. 1A) (10). Stable cell lines containing high levels of replicon RNA and viral proteins proved useful to study HCV replication as well as translation and processing of HCV proteins present in the system (10–12).

To examine the influence of the HCV replicon on the cellular transcription, we utilized microarray technology. Differential hybridization of more than 400 cDNAs with probes derived from RNA of mock- and replicon-transfected HuH7 cell lines resulted in the identification of several de-regulated genes. In the present study we focus on the analysis of the gastrointestinal-glutathione peroxidase (GI-GPx) gene, whose mRNA was down-regulated in the replicon cell lines up to 20-fold compared with control cell lines.

GI-GPx is one of four selenium-containing glutathione peroxidases, and its expression in rodents appears to be restricted to the epithelium of the gastrointestinal tract (13), whereas in humans it is also found in liver (14). Glutathione peroxidases are functioning primarily to counteract oxidative attack but also play roles in signaling (reviewed in Refs. 15 and 16). Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, are produced during aerobic metabolism. Glutathione peroxidases, along with superoxide dismutases and catalases, are considered the main antioxidant enzymes to encounter oxidative stress, which can cause severe cell damage (17). Selenoproteins like GPx incorporate selenocysteine (Sec) co-translationally into the polypeptide chain by a complex mechanism (reviewed in Refs. 18 and 19). Sec has its own code word, UGA, that serves a dual function of dictating cys insertion sequence; UTR, untranslated region; IRES, internal ribosomal entry site; SOD, superoxide dismutase; PBS, phosphate-buffered saline; PKR, RNA-activated protein kinase; siRNA, small interfering RNA; IFN, interferon.
Inverse Expression of GI-GPx and HCV Replicon

We report that, compared with control HuH7 cells, HCV-replicon cells expressed less GI-GPx and were more susceptible toward ROS-producing agents. Ectopic expression of GI-GPx in replicon cells resulted in a decrease of the HCV RNA and protein NS5a. We show that up-regulation of GI-GPx by retinoic acid and selenite caused drastic down-regulation of HCV in the replicon system. Therefore, retinoids, which exert their action by influencing gene networks, may be useful for treating HCV patients.

EXPERIMENTAL PROCEDURES

Cell Culture— Cultures of the human hepatoma cell line HuH7 (23) and the HuH7 cell clones 5-15, 9-13, and 11-7, which contain the HCV replicons 1α,NS3–3′, 1α,NS5–5′, and 1α,NS2–NS3′ (Fig. 1A), respectively, were propagated as described (10, 11). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated, fetal bovine serum (Invitrogen) and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. G418 (geneticin) (Invitrogen) was added to a final concentration of 750 μg/ml to medium used for culturing cell lines carrying HCV replicons. Cells were routinely passaged every 2–3 days at a dilution of 1:2 to 1:4, depending on density of each cell line. Human interferon α was purchased from Sigma. HEK 293 cells were transfected by the Ca2+-GTP/CIT-CCC TCG TAG GTT TAG AGG AAA CAC CCT

primers specific for each type of AtlasTM array. After removal of the scriptase, and a cDNA synthesis primer mix, which is a combination of probe 1 (bp 304–343; GenBankTM accession number Y00435), 5′-GGG GCT CGA GGC CTG AGC CGA GCC CAA CTT ATG-3′; probe 2 (bp 988–1027; GenBankTM accession number Y00433), 5′-GGT CCT CCC TCG TAG GTT TAG AGG AAA CAC CCT CAT AGA T-3′. The 5′-labeled probes were added to freshly prepared pre-hybridization solution at 10 μg/cm² and incubated with the membranes overnight at 65 °C. Filters were washed three times with 2× SSC, 0.2% SDS at 65 °C for 15 min each, followed by three washes with 0.2× SSC, 0.2% SDS at 60 °C for 15 min each. The blots were exposed overnight at −80 °C to Kodak X-Omat AR films with intensifier screens.

Preparation of Cell lysates and Western Blot Analysis— Western blot analyses were performed as described previously (24). HuH7 cells were washed with ice-cold PBS, scraped with a rubber policeman, collected by centrifugation (10 min at 2000 × g), and resuspended in 200 μl of lysis buffer (50 mM Heps, pH 7.5, 0.2 μg/ml NaCl, 1% Triton X-100, 0.4 mM EDTA, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate). The lysed cells were centrifuged for 10 min with 20,000 × g at 4 °C. Protein concentrations of the supernatants were determined by using the BCA reagents (Pierce).

Equal amounts of protein were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA) by semi-dry blotting. The membranes were blocked with PBS/Triton (0.1% Triton X-100 in PBS, pH 7.2), supplemented with 5% low fat milk powder (w/v) for 1 h. The primary antibodies were diluted in PBS, 0.1% Triton X-100 containing 1% low fat milk powder (w/v) and then incubated overnight at 4 °C. We used an NS5 antibody (Biogenesis, Cambridge, UK) (1:1000), a GI-GPx-specific polyclonal antibody kindly obtained from R. Briggerlos-Flohe (University of Potsdam, Germany) (1:500), MnSOD and PKR antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) (1:1000), an α-tubulin and a hemagglutinin tag-specific antibody, both diluted 1:2000 (DAKO, Hamburg, Germany).

After washing, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibody (1:2000 in PBS, 0.1% Triton X-100 with 1% low fat milk powder (w/v) (Dako, Hamburg, Germany) for 2 h at room temperature and washed three times.

Antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) using medical x-ray films (Fuji).

Glutathione Peroxidase Assay— For measuring cellular glutathione peroxidase activity, we followed the manufacturer’s instructions (Calbiochem, Bad Dürkheim, Germany). Pools of drug-resistant cells, harvested with a rubber policeman in 5 mM EDTA, 1 mM dithiothreitol, and 50 mM Tris-Cl, pH 7.5, and lysed by three cycles of freezing and thawing. After spinning for 15 min at 10,000 × g (4 °C), protein concentration of the supernatant was determined with the BCA reagents. 180 μg of protein were applied per assay.

tert-Butyl hydroperoxide was used as substrate, and GPx activity was measured indirectly. Oxidized glutathione, produced upon reduction of the peroxide by GPx, is recycled to its reduced state by glutathione reductase by oxidation of NADPH H+ to NADP+. The oxidation of NADPH+ is accompanied by a decrease in absorbance at 340 nm, which provides a spectrophotometric means of monitoring GPx activity.

The rate of decrease in the absorbance at 340 nm was measured. A 2.0-kb promoter fragment of the gpx2 gene was amplified by PCR utilizing genomic DNA isolated from 9-13 replicon cells and HEK 293 cells. The upstream primer (5'-GGG GAT TTC TTT CTA CTA ACA ATT CTC ATT ATG-3') contained an AscI restriction site, and the downstream primer (5'-GGG GCA GCC CTG AGC CCA CTT ATG-3') contained an XhoI restriction site. This fragment was fused with the luciferase gene, which was cloned into the pcDNA3 vector (Invitrogen) by semi-dry blotting. The membranes were blocked with PBS/Triton, and secondary antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) using medical x-ray films (Fuji).

Cell Viability Assay—For quantification of the degree of cell death in cell culture, we employed the viability assay based on the reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase activity. The assay was performed in 96-well microtiter plates (Greiner, Frickenhausen, Germany) as described previously (24), but Alamar Blue (Roche Applied Science) was used instead of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Eight wells per sample point were analyzed, and each experiment was repeated independently at least three times.

Transfection of HuH7 Cells—A 2.0-kb promoter fragment of the gpx2 gene was amplified by PCR utilizing genomic DNA isolated from 9-13 replicon cells and HEK 293 cells. The upstream primer (5'-GGG GAT TAA TTC CTA CTA ACA ATT CTC ATT TCG-3') contained an AscI restriction site, and the downstream primer (5'-GGG GCA GCC CTG AGC CCA CTT ATG-3') contained an XhoI restriction site. This fragment was fused with the luciferase gene, which was cloned into the pcDNA3 vector (Invitrogen) by semi-dry blotting. The membranes were blocked with PBS/Triton, and secondary antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) using medical x-ray films (Fuji).

The expression of GI-GPx and HCV Replicon was determined using the ECL detection system (Amersham Biosciences) using medical x-ray films (Fuji). The rate of decrease in the absorbance at 340 nm was measured. A 2.0-kb promoter fragment of the gpx2 gene was amplified by PCR utilizing genomic DNA isolated from 9-13 replicon cells and HEK 293 cells. The upstream primer (5'-GGG GAT TTC TTT CTA CTA ACA ATT CTC ATT ATG-3') contained an AscI restriction site, and the downstream primer (5'-GGG GCA GCC CTG AGC CCA CTT ATG-3') contained an XhoI restriction site. This fragment was fused with the luciferase gene, which was cloned into the pcDNA3 vector (Invitrogen) by semi-dry blotting. The membranes were blocked with PBS/Triton, and secondary antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) using medical x-ray films (Fuji).
sequence, was shown to suppress HCV effectively (27), although the 5'-UTR siRNA (UUC UCC GAA CGU GUC ACU U) dTdT had little effect on HCV (28). All siRNAs were synthesized by Qiagen-Xeragon.

For annealing, a 20 μM siRNA solution was prepared, heated up to 90 °C for 1 min, and incubated at 37 °C for 60 min. The day before transfections, 5 × 10^6 HuH7 5-15 cells were plated per well of a 6-well plate. Transfections were carried out with Lipofectamine 2000 Reagent (Invitrogen) in serum-free medium. The final siRNA concentration was 50 nM. After 3 days, transfections were repeated, and cells were incubated for another 3 days in the presence or absence of 1 μM all-trans-retinoic acid and 50 μM sodium selenite. Cultures were rinsed, harvested, and lysed, and 20 μg of protein was analyzed by Western blotting.

**Adenovirus Construction, Purification, and Infection**—The adenovirus used here were all E1- and E3-defective derivatives of adenovirus type 5 (reviewed in Ref. 29). The coding region for GI-GPx (0.7 kb) was amplified by PCR by using an upstream primer containing a HindIII recognition site (5'-GGG CAA CAC GAT TAT GGC TTT CAT TGC CAA GTC-3'), start codon underlined) and a downstream primer containing an XbaI site (5'-GGT CAT CTA GAT ATG GCA ACT TTA AGG AGG CGG TTG-3'). The 3'-UTR (0.3 kb) of the GI-GPx mRNA, containing a SECIS element, was amplified using the upstream primer 5'-GCC CTC GAG ATG TGA ACT GCT CAA CAC ACA G-3' with an XhoI recognition site and the downstream primer 5'-CAAT GCC GGC TTC TCT TTC TAG CAG AGT GCC-3' covering the polyadenylation site (AAUAAA) and containing a NotI recognition site for cloning. RNA isolated from HuH7 cells was reverse-transcribed by random priming and used as template for PCR. The cDNA coding for human GI-GPx was cloned with and without 3'-UTR into the transfer plasmid (pPMT) between the cytomegalovirus immediate early promoter/enhancer and the bovine growth hormone/polyadenylation signal. This expression cassette was inserted into a bacterial plasmid-borne adenovirus genome using recombination in bacteria (30, 31). A cloned version of the novel genome was identified by DNA analysis, and the viral genome was released from the plasmid by restriction enzyme digestion, and virus replication was initiated by transfecting the genome into HEK 293 cells. Virus was amplified in modified HEK 293 cells and purified from cell lysates using CsCl density gradient centrifugation as described (31). A cloned version of the novel virus was quantified by protein content using the conversion factor 1 mg/ml pure viroin protein = 3.4 × 10^8 viral particles/ml (31). The control viruses AdJ5 and AdLuc were described previously (32).

**RESULTS**

GI-GPx Is Down-regulated in Replicon Cells—Interference of HCV with cellular signaling events can be reflected in altered gene expression. Possible influences and dependences of HCV RNA replication and HCV proteins on host cell transcription are accessible to analysis with cDNA arrays. Three replicon cell lines (HuH 5-15, HuH 5-15, and HuH 5-15) (10, 11) were analyzed for alterations in cellular RNA expression patterns. HuH7-pcDNA3 cells are HuH7 cells resistant to G-418 by integration of a neo gene-carrying plasmid (pcDNA3, Invitrogen) and served as control. Radioactively labeled complex cDNA probes were derived from the RNA of these four cell lines and were hybridized with the cDNA array membranes (stress and apoptosis arrays, Atlas™, Clontech). Results from this novel signal transduction microarray analysis revealed significant down-regulation (10–20-fold) of GI-GPx message in the three replicon cell lines (data not shown).

To verify the results obtained by cDNA array screening, we performed Northern blot analyses utilizing 10 μg of total RNA of each cell line and two different radioactively labeled DNA oligonucleotides as probes. The sequences were deduced from the coding region (GI-GPx; probe 1) and from the 3'-UTR (GI-GPx; probe 2) (Fig. 1B). Densitometric scanning of autoradiograms of three independent experiments established that the mRNA coding for GI-GPx is about 10-fold (HuH 5-15) and 20-fold (HuH 5-15 and HuH 5-15) down-regulated in replicon cell lines compared with control cell line HuH7 pcDNA3 (Fig. 1B). We performed Northern blot analyses with RNA isolated from four naive HuH7 cell lines with various numbers of passages (passage 15, 41, 80, and 136) to rule out that due to clonal selection the HuH7 pcDNA3 reference cell line overexpressed the GI-GPx mRNA. These cell cultures expressed similar levels of GI-GPx mRNA as the HuH7 pcDNA3 reference cell line (data not shown). Thus, the low amount of GI-GPx mRNA in the replicon cell lines was linked to the presence of HCV-RNA and/or HCV-proteins.

Furthermore, we wondered whether the cytoplasmic member of the glutathione peroxidase family expressed in hepatocytes, the cellular glutathione peroxidase (cGPx), compensates for the deficiency of the GI-GPx message. Stripping the membranes (Fig. 1B) and hybridizing them with radioactively labeled DNA oligonucleotides specific for human cGPx revealed similar cGPx mRNA levels in control cells and replicon cells (Fig. 1B, lower panels). These data demonstrate that the GI-GPx gene is specifically affected by the HCV replicon.

The level of GI-GPx mRNA (Fig. 1B) was also reflected on the protein level. GI-GPx protein was readily detectable in pcDNA3 cells, was hardly detectable in 5-15, and undetectable in 9-13 and 11-7 replicons (Fig. 1C, upper panel). Furthermore, we studied the expression of MnSOD, another enzyme involved in detoxification of cellular radicals. Western blotting demonstrated that levels of MnSOD protein were similar in the three replicon cell lines and in pcDNA3 control cells (Fig. 1C, middle panel).

Replicon Cells Are Susceptible to Oxidative Stress—We addressed physiological consequences of the transcriptional down-regulation of the GI-GPx gene. Therefore, cellular extracts of pcDNA3 control cells and the three replicon cell lines (HuH 5-15, 11-7, and 9-13) were prepared to estimate activity of the cellular glutathione peroxidase. The data show that indeed the three replicon cell lines exhibited only about half of the GPx activity of the pcDNA3 control cells (Fig. 2A). These findings prompted us to investigate whether the replicon cells were more susceptible toward treatment with radical and oxidative stress-producing compounds. Replicon cell lines and control cells (HuH 5-15 pcDNA3) were incubated for 24 h with various concentrations of paraquat (methyl viologen), and the viability of the cultures was measured using the Alamar Blue assay. Paraquat, a bipyrrolic herbicide that uses molecular oxygen to produce superoxide anion radical and subsequently hydrogen peroxide (33), impaired viability of replicon cells more severely than of HuH7 pcDNA3 control cells (Fig. 2B). The estimated LD_{50} values for paraquat calculated from three independent experiments were 260 ± 50 μM for HuH 5-15, 270 ± 75 μM for HuH 5-15, 310 ± 65 μM for HuH 11-7, and 3 mm ± 120 μM for HuH7 pcDNA3. Thus, the HCV replicon cells were about 10-fold more susceptible to this radical-producing compound. Similar results were obtained with the ROS-generating agent 2,3-dimethoxy-1,4-naphthoquinone (data not shown).

**Effect of Interferon on HCV and GI-GPx Expression**—To explore whether the presence of the HCV replicon was responsible for the down-regulation of the GI-GPx mRNA in HuH7 cells, we treated replicon cells with interferon α (1000 units/ml) to decrease HCV RNA and protein. It is known that incubation of replicon cells with IFNa causes rapid depletion of the viral HCV RNA and consequently of HCV proteins (11). For HCV RNA detection, we hybridized the blots with a radioactively labeled oligonucleotide whose sequence was derived from the neomycin phosphotransferase (neo) gene. This gene is located on both the HCV replicon RNA and the pcDNA3 vector used for establishing the control cell line HuH7 pcDNA3. Accordingly, bands of 9 and 1 kb were detected in replicon cells and in HuH7 pcDNA3 cells, respectively (Fig. 3). IFNa treatment of 5-15, 11-7, and 9-13 cultures for 4 and 5 days caused eradication of the HCV RNA (Fig. 3) and NS5a protein (data not shown) to undetectable levels but had no effect on expression of the neo gene in HuH7 pcDNA3 control cells (Fig. 3). The IFNa-induced

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down-regulation of HCV followed an increasing up-regulation of GI-GPx mRNA in HuH 5-15, 11-7, and 9-13 replicon cells. After 4 days of IFNα treatment, GI-GPx mRNA levels of 9-13 cells almost met the levels of control cells (HuH7 pcDNA3) (Fig. 3). IFNα is known to cause an up-regulation of the IFN-response gene PKR (double-stranded RNA-specific protein kinase). Indeed, transcription of the two PKR-specific mRNAs was increased in all HuH7 cell lines after addition of IFNα (Fig.
Fig. 2. A, glutathione peroxidase activity is reduced in HuH7 replicon cells. HuH7 replicon cells and pcDNA3 control cells were plated and harvested (described in legend of Fig. 1B), and 180 μg of protein of cytoplasmic extract was used for estimation of glutathione peroxidase activity as described under “Experimental Procedures.” The mean change (± S.E.) of extinction at 340 nm reflecting glutathione peroxidase activity for each cell line is illustrated. The replicon cell lines HuH 5–15, 11–7, and 9–13 show lower glutathione peroxidase activity than the control cell line pcDNA3. B, replicon cells are susceptible toward oxidative stress. HuH7 cultures were plated in 96-well microtiter plates (5 × 10⁴ cells/well) and, 3 days after seeding, treated for 24 h with the concentration of paraquat indicated (up to 5 × 10² μM). The cell viability was then determined in triplicates with the help of an Alamar Blue assay and compared with untreated cells (100% viability). Although pcDNA3 control cells (circle) showed an LD₅₀ of about 3 × 10² μM paraquat, the replicon cell lines 5–15 (square), 11–7 (triangle), and 9–13 (rhombus) had LD₅₀ values between 260 and 310 μM. A typical result of three experiments is depicted.

3). Furthermore, the HuH7 pcDNA3 data show that GI-GPx was not a direct IFNα-responsive gene. IFNα treatment activated the innate immune response leading to the complete removal of the HCV replicon within 2 days. The up-regulation of GI-GPx in replicon cells by IFNα after 4 days seemed to be a consequence of the depletion of HCV rather than a direct effect of IFNα signaling (Fig. 3). Taken together, these data revealed an inverse correlation of the HCV replicon and GI-GPx.

Effect of Overexpression of GI-GPx on HCV Replicon—To gain more direct proof that GI-GPx prevents the replication of genomic HCV RNA, we overexpressed the human GI-GPx cDNA in replicon cells. Therefore, the GI-GPx coding region was cloned into expression vector pPM7. To allow efficient readthrough of the stop codon coding for selenocysteine, we fused the 3′-UTR of the GI-GPx mRNA containing the SECIS element to the GI-GPx coding region. To control the effectiveness of these constructs, HEK 293 cells were transiently transfected for 1 day with plasmids containing the GI-GPx coding region with and without the 3′-UTR of GI-GPx. Only the construct with 3′-UTR of GI-GPx produced evident amounts of GI-GPx protein (Fig. 4A). Measuring activities of the transfected cultures disclosed a 50% increase in glutathione peroxidase activity when the GI-GPx +3′-UTR construct was used proving the functioning of the constructs (Fig. 4A).

The two pPM7 GI-GPx constructs (+3′-UTR and −3′-UTR) were recombined into the adenovirus genome, and virus particles were produced. For overexpression of GI-GPx mRNA in all three replicon cell lines, 1000 virus particles per cell were used for transduction (Fig. 4B). As control, adenovirus with the gene coding for the green fluorescence protein was used. After 4 days, cultures were harvested, and expression of the HCV protein NS5a and of GI-GPx was analyzed by Western blotting. Only the construct containing the SECIS element (+3′-UTR) allowed expression at detectable levels. The expression of the GI-GPx gene resulted in a dramatic reduction of the NS5a signal in the cell lines 11-7 and 5-15. The effect was weaker in 9-13 cells probably due to lower GI-GPx levels (Fig. 4B).

Seven days after transduction, expression of GI-GPx was still high and NS5a remained down-regulated in these cells (data not shown). These data demonstrate again the inverse correlation between presence of GI-GPx and HCV replicon. Effect of Retinoic Acid on Replicon—In order to utilize a mechanism to activate the endogenous gpx2 gene, coding for GI-GPx, we examined the promoter of the human gene (GenBank™ accession number AF199441). We identified several putative retinoic acid-response elements in the promoter and in the first intron. Indeed, treatment of the breast tumor cell line MCF-7 with all-trans-retinoic acid (RA), a classical morphogen that can induce differentiation and apoptosis (24, 53), increased both GI-GPx mRNA level and enzymatic activity (34). The induction by RA was specific for the expression of the gpx2 gene, but not of the gpx1 gene coding for the cellular GSH peroxidase (34).

In order to assess potent activators of the gpx2 promoter, we stably transfected naïve HuH7 and 9-13 replicon cells with a construct consisting of the human gpx2 promoter (2 kb) upstream of the luciferase reporter gene. Incubating stable pools of transfectants with several retinoids (9-cis-RA, all-trans-RA, 4-((E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid, 13-cis-RA, methoprene acid, AM-580, and 4-hydroxyphenylethinamide; 1 μM each) for 3 days revealed...
that all-trans-RA was the most potent inducer of the gpx2 promoter (2.5-fold), followed by 9-cis-RA, 13-cis-RA, and 4-hydroxyphenylretinamide (Fig. 5A). The pattern was similar in HuH7 and 9-13 replicon cells (data not shown) and implies that RA receptors, but not retinoid X receptors, were involved in gpx2 promoter regulation.

We then incubated the replicon cell line 9-13 for 3 days with the same retinoids (10 μM each in the presence of 50 nM sodium selenite). Only those compounds efficiently activating the gpx2 promoter (Fig. 5A) were able to induce significant down-regulation of NS5a. All-trans-RA, 13-cis-R, and 4-hydroxyphenylretinamide caused reduction of NS5a by about 80%, whereas methoprene acid and AM-480 had hardly any effect on NS5a expression (Fig. 5B).

In order to elucidate the effect of retinoids on HCV in more detail, HuH7 replicon cells were incubated with increasing concentrations (from 10⁻⁴ to 10 μM) of all-trans-retinoic acid in the presence of 50 nM sodium selenite for 1 week. The GI-GPx mRNA (data not shown) and protein levels increased in a dose-dependent manner (Fig. 5C, lanes 4–9). The half-maximal induction was at 1 μM RA, which is in good agreement with effects mediated by retinoic acid receptors (24). In parallel, we studied the effect of activating GI-GPx expression via RA treatment on the HCV replicon by monitoring NS5a levels. In fact, 1 and 10 μM RA (Fig. 5C, lanes 8 and 9) caused significant down-regulation of the NS5a protein. At this concentration we did not observe any cytotoxic effects of RA either on naive HuH7 cells or on replicon cells. Furthermore, growth curves of all cell lines over 1 week in the presence or absence of 1 μM RA (±50 nM sodium selenite) were indistinguishable from control cultures (data not shown).

Notably, RA (10 μM) without sodium selenite hardly induced GI-GPx protein expression (Fig. 5C, lane 3). This finding implies that selenite has to be present in the culture medium for efficient translation of selenocysteine proteins as already described before for hepatocytes (35). Fig. 5C also shows that selenite itself caused a slight increase of GI-GPx protein levels which, however, was not sufficient to affect NS5a levels (lane 2). Even cultivating the replicon cell lines for several passages (3 weeks) in the presence of 50 nM sodium selenite did not cause down-regulation of HCV (data not shown). The finding that RA caused NS5a down-regulation only in the presence of selenite argues that the effect was indeed mediated by the selenocysteine protein GI-GPx.

To strengthen this theory siRNA experiments were performed. Therefore, we ordered two sets of Gpx2 siRNA (Qiagen) and found that only duplex 1 was efficient in down-regulating the GI-GPx expression (see Fig. 5D, middle panel, lane 7). Replicon 5-15 cultures were transfected with GI-GPx siRNAs and again after 3 days. After incubation for an additional 3 days in the presence or absence of all-trans-retinoic acid (1 μM) and sodium selenite (50 nM), expression of NS5a, GI-GPx, and PKR was analyzed. Although cultures transfected with a non-silencing control siRNA responded to the RA/sodium selenite treatment by down-regulation of NS5a (Fig. 5D, lanes 3 and 4) as the control cells (transfection procedure only) did (lanes 1 and 2), this down-regulation was severely reduced by Gpx2 siRNA (compare lane 8 with lanes 2 and 4). This inhibitory effect was not complete, which may be because not all cells took up the siRNA in sufficient amounts or because minor effects of RA on HCV were independent of GI-GPx. The siRNAs targeting the 5'-UTR and the NS5b coding sequence of HCV served as controls. Although the 5'-UTR siRNA had minor consequences on NS5a levels (Fig. 5D, lane 5) as described before (27), the NS5b siRNA effectively suppressed NS5a expression (lane 6), as shown previously (28). The nonsilencing control siRNA and the 5'-UTR siRNA demonstrate that transfection of the siRNAs shown here did not trigger the IFN response (36). Furthermore, neither RA nor transfection of siRNAs caused induction of PKR expression (data not shown).

The RA Effect on the Replicon Is Reversible—Because it is known that RA can have significant effects on cell cycle and differentiation, we investigated whether the replicon cell lines experienced permanent alterations upon RA treatment. The
5-15 cells were cultivated for four passages (each 4 days) in the presence (H11001) and absence of all-trans-RA (H11002) (Fig. 6). RA caused up-regulation of GI-GPx expression and concomitantly down-regulation of NS5a after 3 days (passage 1) (Fig. 6A, lane 2). The decline of NS5a expression to barely detectable levels was even more pronounced during the next three passages (Fig. 6A, lanes 4, 7, and 10). Removal of RA after one passage (Fig. 6A, +/−) caused reduced expression of GI-GPx and recovery of
Inverse Expression of GI-GPx and HCV Replicon

**Fig. 6.** A, wash-out of retinoic acid causes recovery of HCV. Replicon cells 9–13 were incubated for 4 days with (+RA) or without (−RA) retinoic acid (passage 1; P1). The cells continued to be passaged without (−RA; lane 3) or with RA (+RA; lane 4). In addition, the culture of passage 1 containing RA was subcultured without RA (+/- RA; lane 5). These three cultures (−RA, +RA, and +/− RA) were passaged two more times every 4 days (P2 and P4). Western blotting revealed that long-term treatment with RA drastically reduced NS5a levels (lanes 7 and 10). Furthermore, removal of RA led to a reduction of GI-GPx to undetectable levels and a concomitant recovery of NS5a levels after two passages (lanes 8 and 11). B, the levels of NS5a were quantified by densitometric scanning of the autoradiograms (A). Whereas NS5a levels remained quite constant of control cultures (−RA, circles), the NS5a levels in RA-treated cultures decreased down to 5% of control (+RA, diamonds). Most interestingly, NS5a levels of cultures treated with RA recovered within two passages, when RA was left out (+/-−RA; squares). C, the levels of GI-GPx protein were quantified as described for NS5a (B). Removal of RA (+/-−RA; square) caused rapid decline of GI-GPx levels within two passages. One typical example out of three is depicted.

**Fig. 7.** PKR is not involved in retinoic acid-mediated but is involved in IFN-mediated HCV down-regulation. Replicon cells (9–13) were treated for 4 days with 50 nM sodium selenite (Se), 1 μM all-trans-retinoic acid (RA), or with 1 μM all-trans-retinoic acid together with 50 nM sodium selenite (RA/Se). RA, in combination with sodium selenite, induced a significant increase in GI-GPx level and down-regulation of HCV protein NS5a without induction of PKR. α-Interferon (1000 units/ml, plus 50 nM sodium selenite) (IFN), in comparison, caused NS5a down-regulation by induction of PKR expression. Co, control.

NS5a levels (Fig. 6A, compare lanes 4 and 5). The GI-GPx expression was completely lost after one more passage in the absence of RA (Fig. 6A, lane 8). Densitometric scanning of the Western blot analyses demonstrated first that the RA effect on up-regulation of GI-GPx and down-regulation of NS5a was reversible and second that the expression of NS5a and GI-GPx was strictly and inversely correlated (Fig. 6, B and C).

The RA Effect on the Replicon Is PKR-independent—Furthermore, to rule out that the RA effect on the replicon was caused by known replicon antagonists like interferon-dependent antiviral host response, we investigated the effect of RA on the double-stranded RNA-activated protein kinase PKR (Fig. 7). PKR is a ubiquitously expressed protein kinase that is induced by interferon, growth factors, and different stress signals and constitutes the major IFN-regulated antiviral pathway in mammals. Thus, PKR is a key player in the cellular response to stress (37, 38). RA in combination with selenite (RA/Se) caused drastic down-regulation of NS5a by 80% (Fig. 7, upper panel) but did not induce activation of the PKR gene as did interferon (Fig. 7). On the other hand, IFN caused complete down-regulation of NS5a in the presence of PKR. The up-regulation of GI-GPx after IFN treatment was a consequence of the removal of the replicon from the cells as shown before (see Fig. 3).

As additional control to exclude an IFN response by RA, we investigated the effect of RA on the IFN-responsive genes interferon-1 and interferon-2, coding for the type I IFN receptor (38, 55). Neither the INFAR-1 nor the -2 levels were increased by RA treatment (data not shown).

These data are in favor of a novel PKR-independent pathway, by which RA leads to HCV down-regulation. Using subtherapeutic concentrations of IFN-α alone and in combination with all-trans-RA substantiated this hypothesis. Interferon α caused a dose-dependent down-regulation of NS5a, which was further enhanced by addition of all-trans-RA (data not shown). The finding that RA acts independently of interferon implies the possibility to cure patients who do not respond to interferon standard therapy with RA.
DISCUSSION

The present study demonstrates that human liver cells harboring a subgenomic HCV replicon RNA express drastically reduced levels of gastrointestinal glutathione peroxidase. The inverse correlation between HCV and GI-GPx was revealed by several lines of evidence (summarized in Fig. 8). 1) Cells housing the HCV replicon express 10–20-fold less GI-GPx than cells without replicon. 2) Treating replicon cells with interferon \( \alpha \) caused disappearance of HCV RNA and HCV protein NS5a, as described previously (11). HCV down-regulation was followed by a recovery of GI-GPx mRNA levels (this study). 3) Forced expression of GI-GPx in replicon cells by adenoviral gene transfer induced down-regulation of HCV RNA and NS5a. 4) Activating the promoter of the cellular gpx2 gene (coding for GI-GPx) by nuclear receptor ligand retinoic acid in a time- and dose-dependent manner caused down-regulation of HCV. Wash out experiments demonstrated that this effect of RA on GI-GPx expression and HCV replicon was reversible. The retinoic acid effect depends on selenium and is abrogated by Gpx2 siRNA.

The transcriptional down-regulation of GI-GPx in replicon cells was reflected on protein and enzymatic levels and seemed to be responsible for a 10-fold higher sensitivity than control cells to radical-producing agents like paraquat. It was already shown in knock-out mice that glutathione peroxidase protects against the lethal oxidative stress caused by high levels of paraquat (39). Thus, the GI-GPx down-regulation permits selective killing of HCV replicon cells by paraquat and may open new routes for treatment of HCV-infected patients.

Selenocysteine proteins do not only function to protect against oxidative stress but seem to have other crucial roles in maintaining a healthy physiology of the liver (40, 41). Selenium was proposed to have anticarcinogenic functions, and recently it was shown that in progressed stages of colorectal cancer expression of selenoprotein GI-GPx was decreased (42, 58). Selenium has also been implicated in enhancing immune functions and thereby slowing the progression of AIDS in human immunodeficiency virus-positive patients (reviewed in Ref. 40). Our data now point to a more direct antiviral function of the selenoprotein GI-GPx in HCV infection, and it remains to be seen if the same applies to HIV. Because deficiency in dietary selenium results in decreased levels of selenoproteins, thus compromising biological processes that are maintained by these proteins, it will be interesting to investigate whether selenium lack supports HCV spreading in patients.

Here we show for the first time that the selenocysteine protein GI-GPx plays a crucial function in limiting the HCV replicon in human liver cells, and one might speculate that GI-GPx carries antiviral properties. Our novel findings support the hypothesis that GI-GPx, apart from being a barrier against hydroperoxide absorption, may also be involved in cell growth, differentiation, and ultimately in regulating signal transduction (reviewed in Refs. 16 and 58). In a recent study, it was shown that endogenously produced ROS did not lead to NF-κB activation but instead lowered the magnitude of its activation (43). Therefore, it may be an advantage for HCV to abolish GI-GPx expression in order to prevent an NF-κB-driven anti-
viral host response. Although it is not known how the HCV replicon system interferes with the cellular signal transduction machinery to induce down-regulation of the gpx2 gene, it is conceivable that NS5a is involved. Transfection of NS5a itself seems to be sufficient to trigger the elevation of ROS leading to the activation of several cellular transcription factors like NF-κB and STAT-3 (44). Several studies demonstrated that HCV-infected liver cells in patients (45) and in vitro (46, 47) are characterized by a high level of oxidative stress.

A major contribution to nucleic acid damage is caused by oxidative stress due to H₂O₂ and other endogenous reactive oxygen species (48). Therefore, it is conceivable that the down-regulation of GI-GPx expression is accountable for, first, the high rate of mutations in HCV and development of quasispecies and, second, the development of liver fibrosis and cancer (49). Over a long term period, an inappropriate composition of ROS may be even stronger than in the subgenomic replicon cells, among these were the growth arrest and DNA damage-inducible protein GADD153, the DNA repair protein RAD54, the DNA excision repair protein ERCC1, and the damage-specific DNA-binding protein p48 subunit (4). Another consequence of the down-regulation of GI-GPx may be the promotion of synthesis of HCV proteins. Under conditions of cellular stress, the rate of translation for most cellular mRNAs is reduced. However, mRNAs containing an internal ribosomal entry site (IRES) are selectively translated under these conditions. In particular, translation mediated by the viral IRES is enhanced following prolonged exposure to anoxia (50). Consistent with this model is the observation that antioxidants block replication of HCV in the subgenomic replicon system (46).

As stated above, it is conceivable to cure HCV patients by treatment with ROS-producing agents in order to selectively kill the highly susceptible HCV-positive hepatocytes. Furthermore, our data obtained with the adenoviral gene transduction system or by activation of the endogenous promoter of the cellular GI-GPx gene by retinoic acid demonstrate that enhanced expression of GI-GPx supports the host cell in limiting HCV replication. As shown here in human replicon cells, RA can activate the putative retinoic acid-response element(s) of the gpx2 gene. Increasing the GI-GPx activity to reduce cellular radical levels may form the basis for valuable treatments of HCV patients. The differential regulation and the tissue-specific expression of GI-GPx may allow finding means to up-regulate the GI-GPx protein in HCV-infected liver cells.

It is worth mentioning that more than additive effects in inhibiting the replicon were obtained when RA was applied in the presence of ribavirin or subtherapeutic concentrations of interferon. Therefore, it will be crucial to elucidate and understand in more detail the virus/host cell interaction and the controlling elements regulating expression of GI-GPx.

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