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Hepatitis C Virus Inhibits DNA Damage Repair through Reactive Oxygen and Nitrogen Species and by Interfering with the ATM-NBS1/Mre11/Rad50 DNA Repair Pathway in Monocytes and Hepatocytes

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Hepatitis C virus (HCV) infection is associated with the development of hepatocellular carcinoma (1) and causes lymphoproliferative disorders, including mixed cryoglobulinemia and putatively non-Hodgkin’s B cell lymphoma (2, 3). Chromosomal abnormalities are common in the PBMCs of hepatitis C patients as in most cancers (4). Previously, we have demonstrated that HCV infection induces a mutator phenotype by causing dsDNA breaks (DSBs) (5). We have further reported that HCV induces inducible NO synthase (iNOS) mRNA expression and enhances NO production through the action of the viral structural protein core and nonstructural protein (NS)3, and that NO is responsible for DSBs in most cellular genes (6). To identify the source of reactive oxygen species (ROS), our previous publication (7) showed that ROS is generated from mitochondrial damage-induced oxidative burst, leading to ROS generation. Accumulation of DSBs in HCV-infected cells suggests that a HCV-induced oxidative environment may overwhelm cellular antioxidant and DNA repair mechanisms, leading to chromosomal abnormalities.

Defects in DNA repair genes cause genetic instability, gross chromosomal rearrangements, and accumulation of mutations, leading ultimately to neoplastic transformation. Both homologous recombination and nonhomologous end joining (NHEJ) play a role in the repair of DSBs in mammalian cells (8). The interaction of broken DNA with members of the Rad52 epistasis group, including Rad51, a mammalian homolog of bacterial RecA, initiates homologous recombination repair (8). Following DNA damage, Rad51 is redistributed within the nucleus (9, 10) and induces the ATP-dependent homologous strand-pairing reaction that initiates recombination. In contrast, NHEJ works by nonhomology-dependent ligation of broken DNA ends. DNA-dependent protein kinase (DNA-PK) and its associated proteins Ku70, Ku80, and Xrcc4 mediate NHEJ (11).

Some viral transforming proteins, such as the X protein of hepatitis B virus (12), the E6 protein of human papillomavirus (13, 14), the E4orf3 and E4orf6-E1b55K complex of adenovirus (15), and the Tax protein of human T cell leukemia virus type I (16, 17), disrupt cellular DNA repair, leading to chromosomal abnormalities. Cellular DNA repair proteins prevent potential DNA mutations caused by oxidative damage, but are themselves vulnerable to NO-induced oxidative damage because of their active site sulphydryl, tyrosine, and/or phenol side chains (18–20). The possible association between HCV infection and lymphoma has been controversial (21–23). Nevertheless, the possibility that HCV can induce lymphoma by directly infecting B cells is supported by the observation that transgenic mice expressing the HCV core-NS2
protein would develop lymphoma at a high frequency if the expression of IFN regulatory factor-1 was abolished (24). In addition, the expression of the HCV core and envelope proteins (E1 and E2) in transgenic mice is sufficient to induce lymphoid tumors, such as splenomas (25). The HCV infection of PBMC can potentially cause many deleterious effects on cells, one of which is chromosomal damage.

Suppression of DNA repair mechanisms, coupled with the induction of DNA breaks caused by viral proteins, may enhance the mutation frequency and chromosome rearrangements in virus-infected cells. HCV core protein has been reported to inhibit retinoblastoma mRNA expression (26) and impair cell-cycle regulation in stably transformed Chinese hamster ovary cells (27). The core protein also induces dsDNA breaks (6). Previously, it has been reported that NS3 protein binds directly to ATM, causing inhibition of DNA damage repair (28). From these observations, we hypothesized that HCV induces NO and ROS production, following DNA damages, coupled with the inhibiting NHEJ through defective DNA-sensing function, such as Mre11/Rad50/NBS1 (M/R/N) complex or master regulator of DNA damage ATM activation, in turn, would introduce random chromosomal translocations and deletions, leading to predisposition to immune dysfunction and cancer. In this study, we used an in vitro HCV infection system as well as the expression of individual viral proteins in cells or in transgenic mice to demonstrate that HCV core protein inhibits the formation of the DNA damage sensor protein complex M/R/N as well as ATM activation, leading to the accumulation of DNA damage and hypersensitivity to DNA-damaging reagents. We further demonstrated that core protein inhibits NHEJ and oxidatively damaged DNA repair through binding to NBS1. This study provides insights into the potential deleterious effects of HCV on immune cells.

Materials and Methods

PBMCs

Thirteen HCV-infected individuals and seven healthy individuals were analyzed. The aneuploidy/polyplody was scored separately from translocations/gaps/fragments, because they most likely result from very different mechanisms. The HCV infection status of patients and healthy controls was verified. The demographic information of both groups was comparable, such that age and other disease statuses would not affect the cytogenetic results from the PBMC (Supplemental Table I).

Cell culture

Raji cells were obtained from American Type Culture Collection (Manassas, VA); JT cells, an EBV-transformed B cell line, were established, as previously described (2). They were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 20 and 10%, respectively, FBS. Raji and JT cells were further cloned by single-cell dilution and then used for HCV infection using culture supernatant of a HCV-producing B cell line (SB cells) derived from a HCV+ non-Hodgkin’s lymphoma (2). A control infection using UV-irradiated SB cell culture supernatant was included in all the study provides insights into the potential deleterious effects of oxidatively damaged DNA repair through binding to NBS1. This HCV infection system as well as the expression of individual viral proteins in cells or in transgenic mice to demonstrate that HCV core protein inhibits the formation of the DNA damage sensor protein complex M/R/N as well as ATM activation, leading to the accumulation of DNA damage and hypersensitivity to DNA-damaging reagents. We further demonstrated that core protein inhibits NHEJ and oxidatively damaged DNA repair through binding to NBS1. This study provides insights into the potential deleterious effects of HCV on immune cells.

Karyotype analysis

Metaphase chromosomes were prepared using standard procedures (4). Cells were partially synchronized by colcemid (Gibco BRL, Carlsbad, CA; KaryoMAX Colcemid solution), and chromosome spreads were prepared. For each experimental point, 50–100 metaphases were scored to determine the percentage of aberrant cells and the frequency of aberrant chromosomes. Chromosomal aberrations were defined using the nomenclature rules from the Committee on Standardized Genetic Nomenclature for Mice (see the Mouse Genome Informatics Web site at http://www.informatics.jax.org). Individual metaphase photographs were shuffled and scored blindly for the presence of structural chromosomal aberrations (5).

Spectral karyotyping

Multicolor spectral karyotyping (SKY) was done, as previously described (4). In situ hybridization was performed by combining the SKY paint-labeled DNA pools (Applied Spectral Imaging, Vista, CA), using an SD-300/VD5-1300 spectral imagers/charge-coupled device camera mounted on a Leica DMRXA fluorescence microscope, equipped with a Sutter LS-300 xenon arc lamp and a 1-m liquid light guide. Filter sets included the ASI SKY filter set for simultaneous imaging of Spectrum Green, Spectrum Orange, Texas Red, Cy5 and Cy5.5 fluorescent dyes, and a Chroma 31000 DAPI bandpass filter set. A HCX PlanApo x40/1.25 NA plus x1.6 optovar or PL Apos x100/1.4 NA oil immersion lenses (Leica Microsystems, Deerfield, IL) were used. SKY hybridization was performed, according to the manufacturer’s instructions, mounted with DAPI/Vectashield antifade solution (Vector Laboratories, Burlingame, CA), and analyzed using the SkyVision 1.6.2 software. Seven to 12 metaphases were analyzed for each hepatocyte, splenocyte, or MEF preparation. A breakpoint was scored if it was identified in two or more metaphase spreads by SKY.

Gamma-ray irradiation

Cells were serially diluted into 96-well plates until reaching limiting dilution conditions, and were irradiated with various doses of gamma rays from a 137Cs source, or were left nonirradiated. After irradiation, cultures were grown for 2 wk until cell growth was detectable from a single well, and the frequency of clonable cells was determined according to Poisson's distribution. Percentage of survival was calculated by dividing clonogenic survival at a given dose of irradiation by clonogenic survival of unirradiated cells (29).

UV sensitivity test

Cells were plated in six-well dishes and transfected with individual viral protein-expressing vectors. The cells were irradiated with UV (120 J/m2; Stratallinker2400; Stratagene, La Jolla, CA) at 48 h after transfection. After treatment, cells were trypsinized and collected, and cell viability was determined by propidium iodide and annexin V staining using a commercial kit (Oncogene Research, Boston, MA), according to the manufacturer’s directions. Triplicate samples of each treatment in three independent experiments were assayed.

Micronuclei formation

Cells were seeded on coverslips and incubated for 24 h, and then treated with bleomycin (Sigma-Aldrich, St. Louis, MO) at indicated concentrations or mock treated for 48 h. Cells were fixed with 100% methanol for 1 h or more and stained with DAPI for 5 min. The micronuclei (MN) in cells were examined using a fluorescence microscope. At least 1000 cells were counted for each experiment.

Mice

For animal studies, transgenic mice expressing the HCV core protein of genotype 1b under control of the human elongation factor 1a promoter were generated and bred at the University of Southern California transgenic mouse facility. The primary mouse embryo fibroblasts were prepared from both the core-transgenic mouse and its littermate embryos by trypanosomizing the embryonic tissue and plating the dissociated cells. Institutional Animal Care and Use Committee approved all procedures for animal experiments.

RNA interference using small interfering RNA

Raji cells were transfected with ATM-specific small interfering RNA (siRNA) or NBS1-specific pool siRNA (Upstate Biotechnology, Lake Placid, NY) and incubated overnight at 37°C, as previously described (6). Transfection efficiency was verified to be >80% using a control (non-silencing) siRNA labeled with rhodamine (Qiagen, Valencia, CA). Non-functional siRNA (Ambion, Austin, TX) was also used as a negative control.

Cell-free NHEJ assay

The NHEJ assay was carried out in vitro, as previously described (7). Whole-cell extracts were normalized for total protein levels using the Bio-
Rad protein assay (Bio-Rad, Hercules, CA). Reactions (16 µl) were carried out in 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM Mg(OAc)₂, 80 mM potassium acetate, 2 mM ATP, 1 mM DTT, and 100 µg/ml BSA. Cell-free extracts were incubated for 5 min at 37°C before addition of 5 fmol ³²P-end-labeled DNA. pBSK⁺ duplex DNA (2.96 kb; Stratagene) was linearized with EcoRI, dephosphorylated using calf intestinal phosphatase, and ³²P-end-labeled at 5' end using polynucleotide kinase. Cell extracts were preincubated with the inhibitor anti-human monoclonal Xcc64 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:250 dilution, or wortmannin (0.3 µM; Sigma-Aldrich) on ice for 30 min before shifting to 37°C for 5 min, followed by the addition of ³²P-end-labeled DNA. After incubation at 37°C for 1 h, ³²P-labeled DNA products were depurinated by proteinase K (500 µg/ml) and 1% SDS at 37°C for 20 min and analyzed by electrophoresis through 0.7% agarose gels, followed by autoradiography. Quantification of DNA bands was carried out by a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Background-corrected values of the radioactivity incorporated into the damaged and undamaged plasmids were normalized for the amount of DNA.

Immunoprecipitation and immunoblotting
FLAG-tagged Nbs1 constructs were transfiected into Huh7 cells, and cells were collected for analysis 36 h later. For immunoblots, all cells were Dounce homogenized in buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSE, 0.4 U/ml aprotinin, 1 mM NaF, 10 mM glycerophosphate, and 0.1 mM Na vigos). Equal amounts of protein were immunoprecipitated at 4°C with Abs against FLAG (M2, Sigma-Aldrich), Mre11, Rad50 (both from Novus Biologicals, Littleton, CO), or isotype control Ab (Santa Cruz Biotechnology). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Riviera Beach, FL). Membranes were serially blotted with Abs specific for hemagglutinin (HA; F-7; Santa Cruz Biotechnology), FLAG (M2), Rad50 (Novus Biologicals), Mre11 (Novus Biologicals or Oncogene Research), Nbs1 (Novus Biologicals or BD Transduction Laboratories, San Jose, CA), DNA-PK (sc-9051; Santa Cruz Biotechnology), Ku70 (sc-1486; Santa Cruz Biotechnology), Ku86 (sc-1484; Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). HRP-linked secondary Abs (Amersham Biosciences, Pittsburgh, PA) and ECL (Amersham Biosciences). SuperSignal West Pico Chemiluminescent Substrate, or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) were used to detect bound immunocomplexes.

In vitro kinase assays and immunoprecipitation
In vitro kinase assays for Flag-tagged ATM and endogenous ATM were performed, as described (9).

GST-core protein
GST-core (strain TW; genotype 1b) and GST proteins were purified from Escherichia coli strain BL21. After isopropyl β-D-thiogalactoside induction, glutathione beads were used to trap the GST protein, which was induction, glutathione beads were used to trap the GST protein, which was

M/R/N proteins
M/R/N complexes were purified, as previously described (10, 11). His-Nbs1 expressed with Mre11 or with both Mre11 and Rad50 was eluted from a nickel column using 0.4 M imidazole. Protein concentrations were determined by Bradford assay (Pierce) and confirmed by comparison with protein standards in Coomassie-stained SDS-PAGE gels. Western blotting of Mre11, Rad50, and Nbs1 was performed with Abs anti-Mre11 (Oncogene Research), anti-RAD50 (GeneTex, Irvine, CA), and anti-Nbs1 (GeneTex), respectively, on polyvinylidene difluoride membrane (Millipore, Bedford, MA) using standard immunoblotting techniques.

Gel mobility shift assays
Gel mobility shift assays were performed in a volume of 10 µl with 25 mM MOPS (pH 7.0), 50 mM NaCl, 1 mM DTT, 0.1% Tween 20, 100 µg/ml BSA, 5 mM magnesium chloride, 0.5 mM ATP as indicated, and 1 mM oligonucleotide substrate, with M/R/N or Mre11/Rad50 (M/R) complex added as indicated in the figure legends (between 20 and 120 ng, which is 1.7–10 nM assuming 1.2 × 10⁷ g/mol for M/R/N). Unlabeled dsDNA (T/P=2) was added to the reaction mixture before the addition of protein. Mobility shift assay was carried out by the DNA and other reaction components for 15 min at room temperature before the addition of 1 µl 50% glycerol and separation on a 0.7% agarose, 0.5X Tris borate-EDTA gel at 5.7 V/cm for 100 min. Gels were dried and analyzed using a phosphor imager (Amersham Biosciences). In the competitor assays, the amount of complex formed by M/R/N in the absence of competitor was considered 100%, and the decreases in complex formation relative to that amount were determined by quantitative phosphor imaging analysis.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) assay was performed, as previously described (1). Transient VDJ recombination assays. Assays were performed, as previously described (17). In brief, full-length RAG-1 and RAG-2 expression constructs (18) and the pH290 coding joining or pHJ200 RS joining substrate plasmids (19) were transfected into the cells using FuGENE6 (Roche, Indianapolis, IN). The cells were harvested 48 h after transfection, and the plasmids were isolated by alkaline lysis and then electrophoresed into E. coli MC1061. VDJ recombination efficiencies were determined by calculating the ratio of 250 µg/ml amphotericin- and 20 µg/ml chloramphenicol-resistant colonies to amphotericin-resistant colonies. The fidelity of the RS joins was determined by PCR amplification of the recombining segment of pH200 and subsequent digestion of the products with ApaLI. Coding join sequences was determined by isolating individual recombinant pH290 clones from the double selection plates and nucleotide sequencing.

Statistical analysis
Statistical analysis of the data in aberrant chromosomes was performed by the χ² test. The t test was performed in the DNA damage sensitivity assay. The p values <0.05 were considered to be statistically significant.

Results
HCV infection induces multiple chromosomal aberrations
To determine whether HCV infection induces chromosomal aberrations, we first karyotyped PBMCs from hepatitis C patients. Previously, we have shown that PBMCs from hepatitis C patients often harbor HCV RNA and have a significantly higher mutation frequency in multiple cellular genes (6, 30). In the current study, we further found that PBMCs from HCV-infected patients showed frequent chromosome gaps (Fig. 1A). To characterize the nature of the chromosomal aberrations, we performed SKY analysis (31). Breakpoints on the SKY-painted chromosomes were determined by comparison with the corresponding DAPI karyotype of the same samples (32). Representative metaphase chromosomes revealed multiple translocations involving different chromosomes, such as t(1;19) and dicentric Y chromosome (Fig. 1B). Interestingly, HCV-associated hepatocellular carcinomas frequently have losses or translocations of chromosomes 1, 4, 5, 6, 8, 13, 16, and 17 (33), which partially overlap the pattern of chromosomal aberrations observed in this study. The chromosomal aberrations at these fragile sites may have selective advantages in vivo and thus contribute to HCV oncogenesis. In addition, aneuploidy/polyploidy was frequently observed in HCV-infected PBMC. Because aneuploidy/polyploidy formation involves a different, but overlapped, molecular mechanism (34), it is excluded from this study. Because hepatitis C is a chronic inflammatory condition that causes chronic antigenic stimulation of B cells (35), we also examined PBMCs from non-HCV patients with a different chronic inflammatory condition (Supplemental Table 1). Overall, metaphases from HCV-infected PBMCs showed a much higher frequency of chromosomal aberrations that from HCV− PBMCs or healthy individuals (Fig. 1C, p < 0.05). A breakdown of different chromosomal aberration types and frequency is shown in Supplemental Table 1. The HCV infection status of the patients and healthy individuals was verified by RT-PCR analysis of viral RNA (Fig. 1C), and the demographic information of both groups was comparable. HCV RNA could be detected as late as 180 d postinfection (Fig. 2C), indicating persistent infection. These results indicate that HCV infection is associated with frequent chromosome gaps and translocations in PBMCs.
Occurrence of chromosomal breaks and translocations in PBMCs of HCV patients. 

A. A metaphase from PBMC of a HCV-infected patient. Arrows indicate chromosomal gaps. B. A representative metaphase of HCV+ or HCV− PBMCs from HCV patients and a healthy individual after hybridization with fluorescent probes, showing chromosome translocation t(1;19) and a dicentric Y chromosome. Hybridization signals to specific spectral ranges are identified by colors. C. Frequency of chromosomal aberrations in PBMC of healthy, HCV-infected, and non-infected individuals. Absence or presence of HCV RNA in PBMCs of four healthy individuals, four hepatitis C patients, and three noninfected patients of HCV RNA was demonstrated by RT-PCR of HCV RNA.

To establish that the observed chromosome aberrations were caused directly by HCV infection, but not caused by inflammation, we infected an established B cell line (JT cells) with a lymphotropic strain of HCV (SB strain) (30) in vitro and examined the metaphase chromosomes at various periods of time postinfection. HCV-infected cells displayed a high frequency of chromosomal gaps (Fig. 2A) and other structural aberrations, including quadriradial structures, chromatid gaps and deletions, acentric fragment, and complex rearrangements involving different pairs of chromosome (Fig. 2B). Similar observations were made in another cell line, Raji cells, infected with HCV (Fig. 2C). One of the chromosomal translocations, t(8;14), is an inherent chromosome marker change in Raji cells (36) and was found in both uninfected and infected Raji cells. However, HCV-infected Raji cells contained numerous additional translocations, including t(10;11), t(8;21), and t(9;10) (Fig. 2C). Three independent HCV infections were performed, and similar results were obtained. The number of individual clones and the breakdown of different chromosomal aberration types are shown in Supplemental Table II. The frequency of chromosomal aberrations in HCV-infected cells increased significantly from day 14 to day 180 postinfection (Fig. 2D, 2E, p < 0.05), whereas that of the uninfected cells remained unchanged during the 6-mo culture period. Chromosome loss also occurred frequently during long-term culture of the infected cells. Chromosome losses were noted particularly in chromosomes 5, 14, 16, 17, 21, and 22 of HCV-infected cells (Fig. 2F). The propensity for chromosome loss was greater than that for chromosome gain (Fig. 2F, 2G). As cancer cell lines, such as Raji cells, often have a tendency to lose or gain particular chromosomes, we performed three independent infections on an additional cell line (JT cells). The patterns of chromosomal aberrations seen in the HCV-infected cells varied between different passages, suggesting that chromosomal aberrations were common events, but most of the aberrations did not confer a selective advantage. These observations demonstrate that HCV infection induces genetic instability, causing random chromosomal rearrangements, and that chromosomal aberrations of HCV-infected cells occur in a stochastic manner.

Cotreatment of NO and ROS inhibitors reduces HCV-induced chromosomal damages

Previously, we have shown that HCV-induced DSBs and mutations of cellular genes were caused by NO and ROS production (6). We therefore tested the role of NO and ROS in chromosome instability of the HCV-infected cells. For this purpose, cytogenetic analysis was conducted on the HCV-infected cells that had been treated with iNOS inhibitor (1400W) or N o-nitro-l-arginine methyl ester (l-NMMA) and GSH inhibitor (N-acetylcysteine [NAC]) or ROS inhibitor (buthionine sulfoximine [BSO]) from the beginning of infection (6). These inhibitors significantly lowered the frequency of aberrant metaphases in HCV-infected cells (p < 0.05), while having no effects on the basal frequency of chromosomal aberrations in the uninfected cells or cells treated with each inhibitor alone (Fig. 2H, 2I, Supplemental Fig. 1), indicating a synergistic inhibition between ROS and iNOS-mediated chromosomal aberrations. It is noteworthy that these inhibitors did not prevent the establishment of HCV infection in Raji cells (6). These findings indicate that chromosomal damages in the HCV-infected cells were at least partially mediated by NO and ROS.

HCV infection sensitizes cells to DNA-damaging agent-induced cell killing

Because chromosomal anomalies could be caused by increased DNA damages and/or decreased repairs, we next evaluated the efficiencies of DNA damage-repair mechanisms in HCV-infected cells. It has been shown that yeast or mammalian cells defective in DNA damage repair are more sensitive to various genotoxic agents, including gamma-ray and UV irradiation and bleomycin treatment (37–39). We first assessed colony-forming ability of HCV-infected Raji cells exposed to increasing doses of gamma-ray irradiation. The results showed that the survival rate of HCV-infected cells was at least 50% lower than that of the uninfected cells after gamma-irradiation at various doses, indicating that HCV infection sensitized cells to gamma-irradiation (Fig. 3A).

Similar observations were made on the sensitivity of cells to UV irradiation. Without UV treatment, HCV-infected Raji cells showed 18% apoptosis, which is consistent with the previous finding that HCV infection itself causes a 20% apoptosis rate (30). At every UV dose, HCV-infected Raji cells showed a 2-fold higher percentage of apoptotic cells (as determined by annexin V assay) than that of the uninfected counterparts (Fig. 3B).
We also examined the formation of MN, which are small nuclei arising fromacentric chromatids or chromosome fragments, induced by bleomycin (40). After treatment with bleomycin (500 nM), the frequency of cells with MN was significantly higher in HCV-infected cells than that in the uninfected cells (Fig. 3C). These MNs included chromatid breaks and chromatid exchanges as characterized by triradials and quadriradials (Fig. 3D).

These data combined indicate that HCV-infected Raji cells are defective in DNA-damage repair. We then studied which HCV protein was responsible for such effects. Raji cells transfected with individual HCV proteins were examined for their sensitivity to gamma-ray, UV, and bleomycin treatment. The results showed that, among all the HCV proteins, only Core and NS3 consistently yielded higher sensitivity to these genotoxic agents (Fig. 3E–J). Notably, the core protein yielded the strongest effects in enhancing sensitivity to DNA-damaging agents. However, the expression level of each protein could not be unequivocally quantified; therefore, we cannot rule out the possibility that other viral proteins may also contribute to the increased sensitivity of cells to DNA-damaging agents. In addition, MEFs derived from the core-transgenic mice were also more sensitive to UV than those from the nontransgenic littermates (Supplemental Fig. 2). These results combined indicate that NS3 and notably Core are most likely responsible for the enhanced sensitivity of HCV-infected cells to DNA-damaging agents. In the later sections, we focused on the roles of core protein.

**Enhanced radiation sensitivity of core-transgenic mice**

To establish that the enhanced radiation sensitivity associated with HCV protein expression was not a cell culture artifact, we determined the radiation sensitivity of transgenic mice expressing the HCV core protein. Ten-week-old core-transgenic mice, which ubiquitously express core protein in all tissues, and their nontransgenic littermates were irradiated with different doses of gamma-ray, and the mice were monitored for morbidity and mortality. At 400 rad (Fig. 3K), all nontransgenic mice survived the 4-wk duration of the experiment without morbidity. In contrast, 20% of the core-transgenic mice died between 5 and 8 d after irradiation, in addition to showing diarrhea, peritoneal inflammation, and walking difficulties. At 800 rad (Fig. 3L), 20%
FIGURE 3. Sensitivity of HCV-infected cells to genotoxic agents. A, Sensitivity of HCV-infected Raji cells to ionizing radiation: gamma-rays. The data are expressed as the percentage of surviving colonies in gamma-ray–irradiated cells relative to that in unirradiated cells. At 10 d after irradiation, cells were stained with Coomassie blue, and colonies that contained >50 cells were counted as survivors. The number of colonies on plates seeded with sham-treated cells was corrected for plating efficiency and taken as 100% survival. Points indicate means of three experiments; bars indicate SD. B, UV sensitivity of cells was analyzed by FACS after staining with annexin V at 48 h after UV irradiation. Bars indicate SD. C, The percentage of cells with MN formation with bleomycin for 48 h. Asterisks indicate statistical significance (p < 0.05, t test). D, Representative examples of MN found in HCV-infected cells. E and F, Gamma-ray sensitivity of cells expressing individual HCV proteins. Colony assay was performed as in A. G and H, UV sensitivity of cells expressing individual HCV proteins. I and J, MN formation in HCV protein-expressing cells after bleomycin treatment. K and L, The sensitivity of core-transgenic mice to ionizing radiation. The survival curve of the core-transgenic mice after ionizing irradiation. Mice were irradiated with either 400 (K) or 800 rad (L) of gamma-rays and observed daily. M–P, H&E-stained sections of the small intestine from wild-type (M, N) and core-transgenic (O, P) mice 6 d after irradiation with 800 rad (original magnification ×100).
of nontransgenic mice died at the end of the 4-wk period, whereas as high as 80% of the core-transgenic mice died within the same period (Fig. 3F). These results indicate that core protein expression in mice enhances sensitivity to gamma-ray irradiation.

To determine the cause of death, the irradiated (800 rad) wild-type and core-transgenic mice were examined histologically at 3 and 6 d postirradiation. Highly proliferative cell types, including intestinal cells and bone marrows, are known to be sensitive in response to gamma-irradiation. The core-transgenic mice showed severe signs of acute radiation toxicity in the gastrointestinal tract, including blunted and shortened villi, and obliterated crypts and dilated degenerative crypts (Fig. 3O, 3P), whereas the nontransgenic mice showed normal histology (Fig. 3M, 3N). Thus, the core-transgenic mice are more sensitive than the wild-type mice to gamma-irradiation.

HCV infection inhibits NHEJ

We next determined which DNA repair pathway was inhibited by HCV. We started with the NHEJ pathway because this NHEJ pathway of DNA repair pathway is required for genomic stability and the suppression of translocations (41). Cell-free lysates from the HCV-infected and uninfected Raji and JT cells were examined for their ability to promote in vitro rejoicing of radiolabeled, linear DNA substrates for NHEJ assay (41). Successful NHEJ results in the formation of dimers and higher-order concatemers from substrate monomers. The results showed that HCV-infected cell (both Raji and JT cells: day 10 postinfection) lysates had a lower NHEJ activity than that of mock-infected cells or cells infected with UV-inactivated HCV (HCV− cells) (Fig. 4A, 4B). The DNA dimer formation was completely inhibited by wortmannin and anti-Xrcc4 Ab, consistent with the fact that Ku70, Ku86, and Xrcc4 mediate NHEJ (10). The protein amounts of Ku70, Ku86, and DNA–protein kinase catalytic subunit (DNA-PKcs) were equivalent in these cells, indicating that the mRNA or protein levels of NHEJ were not affected by HCV infection, but their function was inhibited, suggesting that HCV may block repair enzymes at the posttranslational level through ROS and/or reactive nitrogen species (RNS). The expression of core or NS3 protein alone reduced the NHEJ activities, whereas none of the other viral proteins (E1, E2, NS4B, NS5A, and NS5B) did (Fig. 4C). We have previously shown that NO induces DNA damage in HCV Core- and NS3-expressing cells (6). Therefore, we next determined whether NO-mediated toxicity is involved in genotoxic stress sensitivity. The iNOS and Gapdh cDNA amplification under this condition occurred in a linear range, ensuring accurate determination of the ratio of iNOS mRNA to Gapdh mRNA (after normalization). The activation of iNOS mRNA expression was confirmed in core- or NS3-transfected cells (Fig. 4E).

To confirm that the iNOS induction seen is functionally significant, we determined the nitrate and nitrite levels in the culture supernatant of transfected cells. The amount of nitrites and nitrates produced from Core- or NS3-transfected cells was significantly higher than that from mock or other viral protein-expressing cells (Fig. 4D). As a positive control, cells treated with the stimulatory cytokines or the NO donor S-nitroso-N-acetylpenicillamine (SNAP) also produced higher levels of nitrites and nitrates. These results demonstrated that core or NS3 expression enhanced the production of nitrites and nitrates as a result of iNOS activation. Again, the amounts of NHEJ components were not significantly different among cells expressing the different viral proteins (Fig. 4C). Furthermore, cell lines (HEK293 and HepG2) stably expressing the core protein and MEF from three representative core-transgenic mice showed a lower NHEJ activity than those of the control cells and nontransgenic littermates, respectively (Fig. 4F) (data not shown). Immunoblots of Ku70, Ku86, DNA-PKcs, and Core proteins showed no significant difference among key NHEJ components (Fig. 4G). RNase protection assay of NHEJ components in core-expressing cells demonstrated that RNA levels of major NHEJ components were not significantly different between the wild-type and core-transgenic mice (Fig. 4G, lower inset).

To rule out the possible artifacts of the cell-free in vitro assays of NHEJ, we further studied V(D)J recombination, which is mediated by NHEJ (42), in MEF derived from core-transgenic mice. The MEF from core-transgenic mice showed a lower efficiency of V(D)J recombination (Supplemental Table III). These results suggest that HCV infection, through the viral core and NS3 proteins, inhibits NHEJ repair.

HCV-induced NO and ROS production impairs repair of oxidative DNA damage

Because core and NS3 induced DNA breaks through the induction of NO and ROS (6, 7) and NHEJ is inhibited by NO (19, 20, 43), we next determined whether the NO inhibitors restored the function of NHEJ activity. For this purpose, cell-free extracts from HCV Core-expressing Raji cells that had been treated with or without the NO and/or ROS inhibitors were examined for their ability to promote in vitro monomer into a dimer substrate through NHEJ. The results showed that dimer was formed when extracts from HCV-neo cells were used, whereas no dimer was formed using HCV core cell extracts (Fig. 4H). To modulate oxidative stress in Core-expressing cells, the intracellular GSH level was modulated with NAC, a GSH precursor, and BSO, a specific GSH synthesis inhibitor (ROS inhibitor). Lysates from the HCV core-expressing cells treated with an iNOS inhibitor (1400W), NOS inhibitor (L-NMMA), and/or GSH inhibitor (NAC) alone did not recover NHEJ defects, indicating that the inhibition of NHEJ by HCV core protein expression is not mediated through NO or GSH-mediated response alone. Treatment of cells with the 1400W or L-NMMA suppressed the elevation of the nitrite and nitrate levels in the supernatant of HCV core-expressing cell culture (6). Lysate treated with 1400W and NAC had nearly the normal level of dimer formation activity (Fig. 4H). These results suggest that the viral core protein inhibits NHEJ repair in a manner dependent on NO and oxidative stress, but possibly through ROS production.

Core protein induces chromosomal aberrations

Because the inhibition of DNA repair could lead to chromosomal aberrations, we next assessed whether the expression of the core protein alone induced chromosomal aberrations. For this purpose, we used a hepatocyte cell line stably expressing the core protein and a control cell line with a neomycin-resistance gene, HepG2neo. Chromosomal aberrations were charted periodically during a 6-mo interval. Cells were split (1:5) every 4 d. Approximately 8% of HepG2-neo cells had chromosomal translocations at the beginning of the culture period; this frequency remained unchanged during serial passages (Fig. 5D). In contrast, the frequencies of aberrant chromosomes in the core-expressing HepG2 cells increased as cell passage number increased. About 23% of metaphase chromosomes contained aberrations at passage 38 (Fig. 5D). Multiple chromosomal aberrations could be seen in some cells (Fig. 5A). The patterns of chromosomal aberrations among individual metaphases prepared from the same cells were heterogeneous, suggesting that gross chromosome rearrangements occurred continuously during cell passages, but most of the aberrations did not confer a selective advantage. Similar observations were made in HEK293 cells expressing the core protein (Fig. 5C). The cytogenetic data in Fig. 5 are categorized in Supplemental Table II. These aberrations included nonreciprocal clonal rearrangements.
FIGURE 4. The effects of HCV on NHEJ recombination repair. A and B, Extracts from mock- or HCV-infected Raji and JT cells (day 10 postinfection) were incubated with the \(^{32}\)P-labeled plasmid DNA (3 kb, arrow) in the NHEJ assay. The DNA was separated by electrophoresis. The dimer produced by end joining is indicated. Pretreatment with wortmannin or anti-Xrcc4 Ab inhibited the end-joining activity. Quantification of DNA end-joining efficiency was carried out by a STORM PhosphorImager (Molecular Dynamics). Background-corrected values of the radioactivity incorporated into the damaged and undamaged plasmids were normalized for the amount of DNA. The levels of Ku70, Ku86, and DNA-PKcs in the cells were detected by immunoblots. HCV RNA in the infected cells was detected by RT-PCR.

C, NHEJ assay using extracts from cells expressing individual HCV proteins.

D, \(\text{NO}_3^-\text{NO}_2^-\) production in cell culture medium in the presence or absence of 1400W or L-NMMA. For a positive control, cells were treated with a mixture of cytokines (IL-1\(\beta\) at 0.5 ng/ml; IFN-\(\gamma\) at 100 U/ml; and TNF-\(\alpha\) at 10 ng/ml) or 0.3 mM SNAP, releasing endogenous and exogenous NO, respectively.

E, Induction of iNOS mRNA in Core-expressing Raji cells was cultured, and culture media was harvested at day 8 posttransfection. The iNOS mRNA was detected by quantitative RT-PCR.

F, NHEJ assay using splenocytes from three different HCV core-transgenic (Core Tg) mice and nontransgenic littermates (wild-type). The amounts of Ku70, Ku86, and DNA-PKcs were examined by immunoblots. Quantification of DNA end-joining efficiency was carried out as indicated above. G, Immunoblots of Ku70, Ku86, DNA-PKcs, and Core proteins (lower inset). RNase protection assay of NHEJ components in core-expressing cells.

H, Restoration of the DNA repair activity by NOS inhibitors in cells expressing individual HCV proteins. Combination of ROS and RNS inhibits NHEJ. The effects of HCV Core-induced NO and ROS on NHEJ and homologous recombination repair. NHEJ assay using extracts from cells expressing HCV core protein or neomycin phosphotransferase (Neo). Extracts from HCV Core-expressing HepG2 cells or HepG2-neo cells were incubated with the \(^{32}\)P-labeled plasmid DNA (3 kb, arrow) in the NHEJ assay. The DNA was separated by electrophoresis. The dimers produced by end joining are indicated. Pretreatment with INOS inhibitor (1400W), ROS inhibitor (NAC), or anti-Xrcc4 Ab inhibited the end-joining activity. For positive controls, cells were treated with a mixture of cytokines (IL-1\(\beta\), IFN-\(\gamma\), and TNF-\(\alpha\)) or SNAP, inducing endogenous NO (19).
in the age-matched nontransgenic splenocytes (Fig. 5A,5B fold higher in the splenocytes of HCV core-transgenic mice than the percentage of structural chromosomal aberrations was nearly 2-50-wk-old core-transgenic mice. Cytogenetic studies revealed that atocytes, through ROS and iNOS.

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These results indicate that the core protein, by itself, induces chromosomal anomalies, not only in Raji cells, but also in hep-
tocytes, through ROS and iNOS.

We further karyotyped metaphases of primary splenocytes from 50-wk-old core-transgenic mice. Cytogenetic studies revealed that the percentage of structural chromosomal aberrations was nearly 2-fold higher in the splenocytes of HCV core-transgenic mice than in the age-matched nontransgenic splenocytes (Fig. 5F, 5G). Fur-

thermore, MEF of core-transgenic mice also displayed a nearly 2-fold increase in chromosomal aberrations as compared with the age-matched nontransgenic littermates (7 versus 4%; data not shown). NAC, BSO, and 1400W treatment of splenocytes derived from core-transgenic mice significantly reduced total aberrations per chromosomes, which was induced in this in vitro culture pe-

riod (Fig. 5H). These inhibitory effects through NO and ROS may not completely explain chromosomal defects because inhibitor treatment only partially inhibits the chromosomal aberrations, indicating that other mechanisms may also be involved in these chromosomal aberrations. These results combined indicate that the HCV core protein induces chromosomal aberrations in tissue culture as well as in whole animals.

Core protein interacts with NBS1 protein, leading to inhibition of M/R/N complex formation, and prevents ATM activation and signaling in response to DSBs

We next attempted to examine the mechanism of the inhibition of DNA repair by HCV and core protein. We first determined master regulator of DNA damage detector ATM activation in response to gamma-ray irradiation. The results showed that ATM phosphorylation was severely impaired in HCV-infected Raji cells or HepG2 cells expressing core protein (Fig. 6A). Similarly, Chk2 phosphorylation was also severely inhibited in the HCV-infected cells and core-expressing cells (Fig. 6A). ATM phosphorylation is triggered by the phosphorylation of the NBS1 protein following the formation of M/R/N complex (44). We therefore examined the protein expression levels of these components. NBS1, Rad50, and Mre11 protein levels were comparable between HCV-infected and uninfected Raji cells (Fig. 6B) and also between HepG2-core cells and control HepG2 cells expressing neomycin-resistance gene (Fig. 6C). To determine whether binding of the Core protein to NBS1 and the inhibition of the assembly of the M/R/N complex is indeed specific for the Core protein, other HCV proteins were examined. NS4B, NS5A, and NS5B did not interact with NBS1, demonstrating the specific interaction between NBS1 and Core protein (Fig. 6D).

We next examined whether the binding of core to NBS1 will interfere with the phosphorylation of NBS1. Huh7 cells were transfected with FLAG-ATM and NBS1, and irradiated with gamma-ray. NBS1 was precipitated by anti–Flag-ATM. Fig. 6E showed that phosphorylated NBS1 was coprecipitated and its amount was significantly decreased in the cells expressing core protein. We further characterized whether Mre11 complex for-
mation was inhibited by the viral core protein. For this purpose, the three proteins of the M/R/N complex were expressed in cells by expression plasmids and then precipitated from cells following gamma irradiation by Ab against NBS1 and subjected to immu-
noblotting analysis with different Abs against various components of the M/R/N complex. The results showed that Rad50 and Mre11 were coprecipitated, indicating the formation of M/R/N complex.

FIGURE 5. Chromosomal aberrations induced by HCV core protein in vitro and in vivo. A and B, SKY analysis of typical metaphase chromosomes from cells expressing core proteins (293core and HepG2core) showing acentric exchange between multiple chromosomes. C and D, Histograms of the cumulative percentages of cells containing aberrant chromosomes at different cell passage levels. E, Frequencies of chromosomal aberrations in HepG2 cells expressing core protein at passage 36 incubated with GSH inhibitor (NAC), ROS inhibitor buthionine sulphoximide (BSO), and/or iNOS inhibitor (1400W). Cotreatment of GSH inhibitor and iNOS inhibitors reduced chromosome aberrations in HCV-infected HepG2 cells. Three independent HCV infections were performed. F and G, Representative examples of chromosomal aberrations in splenocytes from HCV core transgenic mice. Note the extensive chromosomal rearrangements. H, Frequencies of chromosomal aberrations in primary splenocytes expressing core protein versus nontransgenic littermates at 2 wk incubated with GSH inhibitor (NAC), iNOS inhibitor (1400W), and/or ROS inhibitor (BSO) in vitro culture. Cotreatment of ROS scavenger and iNOS inhibitors reduced chromosome aberrations in splenocytes expressing HCV core protein. Three independent HCV infections were performed.

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FIGURE 6. Core protein interacts with NBS1 protein, leading to deficient ATM phosphorylation in response to DNA damages. **A**, ATM phosphorylation upon gamma-ray irradiation. HCV-infected Raji (day 12 postinfection; left), HepG2 cells stably expressing core protein (middle) in the presence or absence of HCV infection (right), were irradiated by gamma-ray and subjected to immunoblotting with ATM- or phospho-specific ATM Abs and chk2 or phospho-specific chk2 Abs. **B**, Protein levels of NBS1, Rad50, and Mre11 in HCV-infected Raji cells. Immunoblot of β-actin serves as a loading control. **C**, Core protein binds to NBS1. HepG2 cells stably expressing core protein were irradiated by 500 rad gamma-ray and immunoprecipitated with anti-core Ab. The immunoprecipitates were blotted with NBS-1, Rad50, Mre11, core, or β-actin Ab. **D**, Individual viral proteins were tested for interaction with NBS1 by immunoprecipitation immunoblots. **E**, Phosphorylation of NBS1 by ATM. Huh7 cells transfected with NBS1 and Flag-ATM were treated with 500 rad ionizing radiation and labeled with [32P]orthophosphate for 30 min. The immunoprecipitated NBS1 was analyzed by autoradiography (top panel) and by immunoblot (bottom panel). **F**, Core protein inhibits the M/R/N complex formation. HepG2 cells stably expressing core protein were immunoprecipitated with anti-NBS1 Ab. The immunoprecipitates were reacted with NBS-1, Rad50, Mre11, or β-actin Ab. **G**, Phosphorylation of NBS1 by ATM. Raji cells were used as described in **E**. **H**, Core protein inhibits the M/R/N complex formation. Raji cells were used as described in **F, I**, Expression of various NBS1 deletion mutants. Schematic representation of the domain organization of human NBS1 and of various NBS1 deletion mutants (d1–d7). The BRCT domain (residues 105–190), and the MRE11 binding domain (residues 601–700) are indicated by boxes together with the ATM phosphorylation sites (Ser278 and Ser343). **J**, Coimmunoprecipitation analysis of the interaction of NBS1 mutants in Huh7 cells. Lysates of cells transiently transfected with a vector for HA-tagged wild-type (WT) or mutant NBS1 proteins (or the corresponding empty vector) were subjected to immunoprecipitation (IP) with Abs to HA-tag, and the resulting precipitates (as well as cell lysates) were subjected to immunoblot analysis with Abs to core or HA proteins. **K**, HCV core protein was detected by Ab against core protein in lysates of eluates from GST-core or GST proteins from E. coli. **L**, HCV core inhibits DNA-binding properties of M/R/N. Gel mobility shift assay was performed with M/R/N or M/R in the presence of HCV core protein, 32P-labeled dsDNA or ssDNA substrate. The 100-fold excess (mol nucleotides) of competitor DNA was mixed with the 32P-labeled DNA before addition of protein. Oligonucleotide substrate (1 nM) was added with M/R/N or M/R complex between 20 and 120 ng, which is 1.7–10 nM, assuming 1.2 3 106 g/mol for M/R/N. **M**, Dose-dependent inhibition of complex formation between M/R/N and dsDNA by recombinant Core protein using gel mobility shift assay. Intensity of bands was quantified. **N**, Hypothetical model of core-mediated repair defects through ATM-M/R/N pathway. **O**, Postulated mechanism of HCV core-mediated NHEJ inhibition through ROS and RNS.
The complex formation was partly inhibited in cells expressing core protein (Fig. 6F); in contrast, the amounts of Rad50 and Mre11 were significantly reduced (Fig. 6F). These data demonstrated that Core protein interacts with NBS1, thus blocking the binding of M/R to NBS1 (the formation of the DNA-damage-sensing complex). Same results were obtained in Raji cells (Fig. 6G, 6H). Taken together, these results show that the HCV Core protein causes inhibition of the M/R/N complex formation to prevent the ATM activation following DNA damage.

To determine which domain of NBS1 is required for the binding with core protein, expression vectors for various deletion mutants of HA-tagged NBS1 were transfected into HepG2 cells (Fig. 6I). HA-tagged NBS1 proteins were immunoprecipitated and immuno-blotted with anti-core and anti-HA Abs. All of the HA-tagged NBS1 proteins of expected size were immunoprecipitated and immuno-blotted with anti-core and anti-HA Ab. Notably, all of the mutants of NBS1, except deletion mutant 6, were coprecipitated with core protein (Fig. 6J). These results indicate that core protein interacts with aa 701–753 of NBS1 protein. These results combined suggest that core protein binds to NBS1 protein, and prevents the formation of M/R/N complex formation in response to DNA damage. As a result, ATM activation (phosphorylation) is inhibited, resulting in deficiency of DNA repair.

To determine whether the interaction between core and NBS1 abrogates the DNA-binding ability of M/R/N complex, a gel mobility shift assay was performed using purified M/R/N complex and HCV proteins (Fig. 6K). In the absence of core protein, M/R/N complex bound to dsDNA (Fig. 6L, lane 2). In contrast, addition of GST-core protein (Fig. 6H) prevented DNA binding of M/R/N (Fig. 6L, lane 3). The 100-fold excess cold DNA probe inhibited the DNA binding of M/R/N, suggesting the specificity of DNA-binding property (Fig. 6L, lane 5). Furthermore, M/R/N complex bound only dsDNA, but not ssDNA, consistent with a previous report (45). Finally, GST-core inhibited the binding of M/R/N complex to dsDNA in a dose-dependent manner (Fig. 6M). These results combined indicate that HCV core protein interacts with NBS1 protein, and thereby disrupts M/R/N complex formation, leading to inhibition of DNA binding of M/R/N complex (Fig. 6N).

**HCV core protein inhibits histone loss from DSB sites and NBS1/ATM-dependent DSB repair**

To further determine the connection between DNA damage response and defective DNA repair, we further performed an experiment to assess the dynamics of the DNA repair protein recruitment and DSB repair under the condition when the NHEJ repair predominates. To assess the corresponding time course of DSB generation and repair, we used an established method by monitoring the time course of I-Ppol-induced DSBs (1). DSBs were induced by 4-hydroxytamoxifen and monitored by quantitative real-time RT-PCR analysis (1) (Supplemental Fig. 4). DSBs were detected as early as 2 h after 4-hydroxytamoxifen induction in conjunction with ATM recruitment and histone H2B loss, whereas DSB repair was detected at 8–10 h in conjunction with XRCC4 recruitment to the DSB site (1). The persistence of DSBs up to 24 h after break induction seems to reflect persistence of active, nuclear-localized I-Ppol enzyme that retains the ability to recombine accurately repaired DSB sites (1). Cells lacking full-length NBS1 protein had detectable, but low, levels of ATM activation after I-Ppol induction (1). However, no ATM or activated ATM could be detected at DSBs by ChIP assay in the absence of full-length NBS1. The re-expression of wild-type NBS1 in these cells resulted in enhanced ATM activation and the recruitment of activated ATM to DSBs (1). NBS1, a component of the analogous MRN complex in mammalian cells, was required for H2B loss at I-Ppol–induced DSBs (1). Recruitment of XRCC4 was also significantly delayed in the absence of full-length NBS1 (1). Lack of ATM also resulted in reduced and asynchronous loss of H2B together with delayed recruitment of XRCC4 to DSB sites (Supplemental Fig. 4A, lanes 10 and 11), which is consistent with a previous report (1). ATM-null cells also had a defect in DSB repair, as reflected by higher DSB levels in ATM-null than in wild-type cells (Supplemental Fig. 4A, lanes 11–13). Same results were obtained in Raji cells (Supplemental Fig. 4D, 4E). To exclude the possibility that these differences in DSB repair in AT cells reflect the use of nonisogenic cell lines, the results were verified by using siRNA-mediated ATM depletion from Huh7 cells (data not shown). ChIP analysis of cells expressing core protein showed that core protein expression inhibits DNA repair complex formation in the damaged sites (Supplemental Fig. 4F, 4E, lanes 6 and 7). ChIP analysis of cells lacking ATM activity reinforced the observation that nucleosome disruption and XRCC4 recruitment depend on ATM activity (1). Unrepaired DNA damage is maintained even after 12 h post-DNA damage in the cells expressing core proteins (Supplemental Fig. 4C, lanes 5–7, 4H, lanes 6 and 7). Complementation of NBS1-deficient cells with NBS1 restored I-Ppol–induced histone H2B loss, as well as recruitment of XRCC4 after induction of DSBs (Supplemental Fig. 4G, lanes 12–14). Using a real-time PCR-based DSB repair-defect assay, higher levels of persistent DSBs were also detected in the absence of functional NBS1 or in the presence of core protein compared with cells reconstituted with NBS1, indicating restoration of repair in cells expressing core protein by overexpression of NBS1 (Supplemental Fig. 4G–I). Same results were obtained in Raji cells (Supplemental Fig. 4J, 4K). These results indicate that core protein inhibits repair complex formation on the damaged sites possibly through ATM and NBS1.

**Discussion**

The studies presented in this work have demonstrated that HCV infection reduces the cell’s ability to repair DNA damage and increases the frequency of gross chromosomal rearrangements, which may cause loss of heterozygosity at functionally important genetic loci. The excessive occurrence of structurally abnormal chromosomes in cells infected with HCV and their accumulation during long-term in vitro culture provide evidence for defective DNA repair associated with HCV infection. We further showed that HCV infection or the expression of viral core protein alone is sufficient to induce spontaneous gross chromosomal alterations in primary cells. The full-length core protein is localized in the cytoplasm, but the various C terminus truncation mutants have been found in the nucleus (46, 47). Because the DNA repair enzymes are localized in the nucleus, the truncation mutants of the core protein are most likely responsible for these effects. We used a unique B cell HCV infection system (PBMCs) to demonstrate the role of HCV infection, especially HCV Core protein, in defective DNA repair. These DNA breaks and translocations in B cells may perturb immune systems. We found that these effects of HCV also apply to hepatocytes.

**Lymphoproliferative disorders induced by HCV may impair immunity**

The lymphoproliferative disorders may be caused by the direct infection of B cells by HCV, and/or by Ag stimulation, which may be followed by the loss of Ag dependence of proliferating B cells and the development of lymphoma (48). The possible association between HCV infection and lymphoma has been controversial (21–23). The largest study, which examined data of 150,000 HCV patients, showed that HCV infection increased the risk of...
lymphoma by only 20–30% (21). The adjusted hazard ratio was 1.28 (21). Another large study, however, found no statistical association between HCV infection and lymphoma (22). HCV infection does not increase the risk of lymphoproliferative disorder in liver transplant patients, although HCV viral load is ∼20-fold increased in these patients. This lack of increased risk was evidenced by the fact that lymphoproliferative disorders associated with HCV infection are not caused by the effects of HCV proteins in infected cells, but rather by chronic antigenic stimulation (23).

Nevertheless, the possibility that HCV can induce lymphoma by directly infecting B cells is supported by the observation (24, 25). We found that HCV, or core protein alone, induces chromosome abnormality through inhibition of DNA-damage repair, as a result of NO and/or ROS production. As a corollary of these findings, our study will suggest that an inflammatory response caused by viral hepatitis, which also induces ROS and RNS through inflammation, may also inhibit NHEJ. These ROS/RNS-mediated inhibitory effects on NHEJ and defective DNA damage-sensing mechanisms (defective ATM-NBS1 pathway) by core protein may synergistically contribute to lymphoproliferative diseases, ultimately leading to the development of lymphomas. For example, t(1;4;18) and t(11;14) chromosome translocation enhances expression of Bcl-2 and c-Myc, respectively, and inhibits apoptosis of immune cells, leading to immune dysfunction and cancer development (49–51). These evidences suggest strong linkage between immune dysfunction and cancer development.

Oncogenic RNA virus HCV may employ a “hit and run” mechanism

Cytogenetic damage represents one of the earliest events in the process of oncogenic transformation. HCV core and NS3 proteins most likely play a key role in this process by suppressing DNA repair of both exogenous and endogenous DNA damages. In this study, we showed that these proteins inhibit NHEJ and oxidative-damaged DNA repair (Supplemental Fig. 4). As a result, HCV-infected cells accumulated multiple mutations (20) and chromosomal translocations. We have previously shown that these two proteins also induce DSBs in a NO-dependent manner and cause enhanced mutation of cellular genes (6). The combination of enhanced DSBs and decreased DNA repair results in chromosomal aberrations, which, in turn, causes activation of proto-oncogenes or suppression of tumor suppressor genes, leading to hepatocellular carcinoma and lymphoma. This postulated chain of events is consistent with the previous reports that the overexpression of core and NS3 proteins induces the transformation of NIH3T3 cells (21, 22). A previous study has also shown that core-transgenic mice have an increased frequency of tumor development (23). Therefore, HCV can potentially transform cells by a “hit and run” mechanism, modifying the host cell genetic materials, resulting in chromosomal aberrations even in the absence of viral sequence (24). Indeed, defects in the components of NHEJ, such as Xrcc4 and Ku80, frequently induce chromosomal translocations (25, 26).

Different chromosomal regions have different susceptibility to the action of oncogenic viruses. Human chromosomes contain fragile sites susceptible to different damages, resulting in specific chromosomal rearrangements associated with different human tumors (27). In addition, defects of NHEJ can erode telomere sequences (28). Telomere dysfunction induces chromosome translocation and accelerates aging (30), which may lead to frequent translocations in HCV-infected cells. Therefore, it is not clear why chromatid breaks and radial chromosomes, which normally occur as a result of defects in homologous recombination (31), could occur in HCV-infected B cell lines. The nature of MN seen in the HCV-infected cells also remains to be studied. Effects of HCV infection on homologous recombination remain unclear. We have examined Rad51 foci-forming activities, but no significant difference between HCV-infected and uninfected cells was observed (Supplemental Fig. 3). Core-induced oncogenesis may be cooperated with other viral proteins NS5B, which might regulate retinoblastoma protein (29), or NS5A, which regulates p53 (52) and PKR (45), leading to HCV-associated malignant liver cancer.

HCV-induced NO induces DSBs and inhibits oxidative DNA damage

The ability of core and NS3 to cause DSBs (3) and inhibit cellular DNA repair can explain the HCV-induced mutations of cellular genes (3). These two proteins induce NO, which, in turn, damages genomic DNA and suppresses DNA repair, the latter of which was most likely caused by the inactivation of DNA repair enzyme active sites by nitrosylation (53, 54). The latter effect will lead to the suppression of base-excision repair (32, 53, 54). HCV-induced DSBs themselves may also inhibit nucleotide excision repair (33). Significantly, the repair activities for gamma-ray–, UV–, or bleomycin-caused DNA damages were all inhibited by core and NS3 proteins. NO may alter the phosphorylation status of the repair enzymes and inhibits their function (54). In this study, however, posttranslational modification of repair enzymes was not investigated in detail.

Inhibition of M/R/N complex formation abrogates the ATM-dependent dsDNA break repair

ATM and M/R/N complex are required for the early G2/M checkpoint in response to ionizing irradiation (34, 35) and prevent DSB accumulation during chromosomal DNA replication (36). Recent studies demonstrated that M/R/N complex plays a critical role in DSB repair through ATM activation (1). The Ku70, Ku80, and DNA-PKcs are involved in the NHEJ (37). The core protein may interrupt the binding of M/R heterodimer to NBS1. Indeed, the immunoprecipitation experiments demonstrated that the proteins coprecipitated with NBS1 did not include Rad50 nor Mre11, indicating that core-NBS1 binding blocked the binding of M/R with NBS1-Core complex. The core protein may sequester the endogenous NBS1 to interfere with the interaction between NBS1 and M/R. Indeed, overexpression of NBS1 in core-stable transforms restored the XRCC4 recruitment to damaged DNA sites (Fig. 6O). Although core protein expression does not affect the expression level of key components of NHEJ, including Ku70, Ku86, and DNA-PKcs, we cannot exclude the possibility that there are direct interactions or functional inhibition of NHEJ components (such as DNA-PKcs or ligase IV) by the core protein. Further study is required for the mechanism of NHEJ inhibition.

In conclusion, HCV, through its core and NS3 proteins, caused chromosomal instability via several mechanisms (Fig. 6O). First, it induces NO and ROS production, thereby inhibiting NHEJ and leading to chromosomal translocations and deletions. Second, binding of core protein to NBS1 inhibits ATM activation. The reduced ability of HCV-infected cells to efficiently repair DNA damage, coupled with the ability of HCV to induce DNA damages, would introduce random rearrangements into the genome, leading to predisposition to cancer. Previously, it has been reported that NS3 protein binds directly to ATM, causing inhibition of DNA damage repair (28). Thus, the combined effects of core and NS3 proteins induce DNA damages, causing either oncogenesis or immune dysfunction.

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