dsRed RNAs and the transduced AdV genome were measured using real-time PCR as described in the Materials and methods section. The ratio of the dsRed RNA level corrected according to the transduced AdV genome between the two cell lines was calculated. The result showed that the dsRed RNA ratio of HuH-7 and SK-Hep1was 42.0:1, correlating well with the quantification of dsRed expression using FACS analyses. Identical experiments were performed using a control AdV AxCAdsRed expressing dsRed under the control of the CAG promoter instead of the double-unit vector. The result showed that the RNA ratio corrected according to the viral DNA amount between HuH-7 and SK-Hep1was 1.38:1, indicating that the activity of the CAG promoter was similar in both cell lines. These results indicated that the 'leak' level of the double-unit vector in the SK-Hep1 cells, compared with that in the HuH-7 cells, was ~1/40th of the expression level of HuH-7 cells (or 1/30th, based on of the CAG promoter control), demonstrating that the background level of double-unit vector in AFP-negative cells was again very low.

To examine the effect of combining the switch unit and the target unit into a single genome, we newly constructed two AdVs: AxLR16EL, containing only the target unit (Figure 7a, first), and Ax-AC, containing the switch unit at the E4 position (Figure 7a, second), as in AxLR16EL-AC. Then, the expression of the double-unit vector (AxLR16EL-AC) and the double infection of split viruses containing the excisional expression unit (AxLR16EL + Ax1AC) was examined using dsRed fluorescence. The double-unit vector showed a much higher fluorescence level than the double-infection method, as observed under a fluorescent microscope (Figure 7b). And the quantitative measurement of dsRed fluorescence showed that the former method produced 3.3-fold more dsRed protein than the latter method using an MOI of 13 (Figure 7c). The reason that the expression level was higher than that of the split viruses appears to be not only because the amount of the target virus was one half of the same total dose of the viruses, but also because in many cells, the split two vectors were not infected simultaneously or did not produce a sufficient amount of Cre during five days.

The steady-state levels of expressed dsRed RNA measured using real-time PCR in HuH-7 cells were compared with the direct expression under the control of AFP promoter (AxA2AdsR, Figure 7a), a double-unit vector (AxLR16EL-AC), a double-infection of split viruses (AxLR16EL + Ax-AC), and the expression under the control of EF1α promoter (AxEFdsR, Figure 7a) (Table 3). On Day 3, the double-unit vector expressed ~40-fold more dsRed RNA than the direct expression under the control of AFP promoter. Meanwhile, the double infection of split viruses (AxLR16EL + Ax-AC) expressed dsRed RNA at a level only 1/4th of that expressed by the double-unit vector, confirming the expression results (Figure 7c). Because the dsRed RNA expressed by the EF1α promoter (AxEFdsR) in HuH-7 cells was 480-fold higher than the direct expression of AFP promoter, the double-unit vector utilized ~1/10th of the EF1α promoter activity on day 3. However, on day 4, the

Table 3. Steady-state levels of expressed dsRed RNA in HuH-7 cells

AdV[a]	Ratio[b]
AxA2AdsR	1
AxLR16EL-AC	41
AxLR16EL + Ax-AC	10
AxEFdsR	480
AxLR16EL-AC (day 4)	91

Each experiment was performed twice to confirm the reproducibility; typical data are shown. The genome structures of the above viruses are shown in Figures 1 and 7a.
[a]HuH-7 cells were infected with each AdV at a MOI of 30. In the double infection, an MOI 15 of each virus was used.
[b]For each condition, the expressed dsRed RNA measured using real-time PCR was divided by the value obtained using the AxA2AdsR virus.

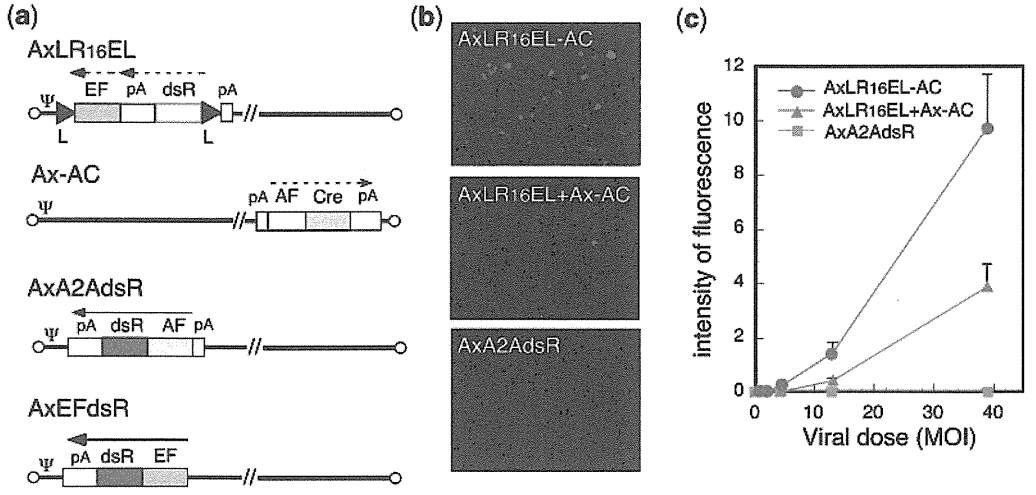

Figure 7. Simultaneous excisional expression in HuH-7 cells using double-infection method. (**a**) Structure of AdVs. The representations are the same as in Figure 1. (**b**) Images obtained using fluorescent microscopy. (**c**) Fluorescence measured using a fluoroscan plate reader.

expression of dsRed RNA reached ~90-fold when using the double-unit vector (Table 3); because such an increase in expression on later days was often observed in other double-unit experiments (data not shown), this result can likely be explained by the weak AFP promoter, since the very small amount of expressed Cre likely required a long time to process the target unit.

DISCUSSION

We developed a 'double-unit' AdV bearing an 'excisional-expression' structure and established a preparation method for this AdV. The AdV showed a high level of expression of the target gene under the control of a tissue/cancer-specific promoter, maintaining a very strict specificity. We observed that (i) because the vector was a first-generation AdV, it could be prepared on a large-scale without difficulty, and (ii) although a leak in Cre expression was observed during its preparation in *E. coli* and 293 cells, both problems were solved using a dominant-negative of Cre, dnCreRY, and an shRNA against Cre, shCreD, respectively. The complete suppression of Cre expression in 293 cells has been especially problematic, since AdV genome replication produces up to ~100 000 genome copies in each 293 cell. The key step in the production of the double-unit AdV was the selection of a clone lacking the AxL-AC virus (Figure 5b, lower left). Our results showed that severe AxL-AC generation caused by the leaky expression of Cre occurred 'before' the selection of a clone lacking the AxL-AC virus and that once such a clone was obtained, problematic deletion caused by 'leaked' Cre was not observed during the second to fourth production steps. Therefore, for the production of double-unit AdV even using 293shCreD13 cells, popular AdV-production protocols producing a pool of AdVs after transfection cannot be used.

As stated in the 'Introduction' section, this vector system is obviously superior to the 'double-infection' method (3); in the latter system, two viruses must be simultaneously transduced into a single cell for expression, and the infection of only one virus in a single cell is useless but causes similar viral toxic effects. Therefore, this new system will be particularly useful under diluted conditions, such as in animal experiments for basic research (unpublished data) as well as human gene therapy. Here, AFP promoter was used as one example, but obviously any tissue/cancer-specific promoter can be used. We intentionally did not adopt a CMV or CAG promoter, but instead used an EF1α promoter as a potent and non-specific promoter in the target unit because we have previously shown that the EF1α promoter hardly induces any inflammation as a result of AdV infection in an *in vivo* experiment, since no detectable induction of viral pIX production occurs (29). One report describing 'excisional expression' where a gene product was expressed only after excision by Cre from a Cre-excised circular molecule as described here has been previously published (30), but the purpose of this previous report was entirely different from that of the present work because the objective of the previous study was to examine persistent expression as a circular replicon using EB-viral oriP and used a double infection system using Cre-expressing AdV. Also, Yant *et al.* (31) reported another type of the 'excisional expression', where intron-containing transgenes were split and thus remained inactive until an FLP-mediated circularization restored the correct reading frame. The excision of an expression unit from the AdV genome by Cre or FLP has occasionally been reported (for examples, 32–34).

We observed a 'leak' in the expression level of the double-unit vector in an AFP-negative cell population amounting to ~1/40th of the expression level in HuH-7 cells measured using FACS as the total sum of all cell florescence, as stated in the Results section. While this level was very low, it was similar to the activity of the authentic AFP promoter in HuH-7 cells (Table 3). However, in AFP-negative cells infected with a double-unit virus, a small number of 'bright cells' highly expressing dsRed (Figure 6b, e and c, f) were present, increasing the apparent 'leaked' expression level.

Importantly, the specificity of the tissue/cancer promoter in this vector was very strictly maintained for a number of reasons. (i) Because the potent EF1α promoter in the target unit is present 'downstream' of the cDNA (Figure 1), an expression leak of the cDNA via this promoter is not possible until the promoter is translocated in front of the target cDNA by *lox*P recombination and circularization. (ii) The *lox*P-combined virus AxL-AC generated during the preparation of the double-unit virus does not contain an expression unit and hence does not cause non-specific expression. In contrast, the double-infection method always generates some stuffer-less, *lox*P-combined virus that causes non-specific expression because a small amount of such virus is generated even without Cre gene in AdV preparations, possibly through the homologous recombination of ~50 nt consisting of *lox*Ps and its surrounding sequences (3). (iii) Though conventional viral stock contains a small amount of circular DNA expressing the target gene and causing non-specific expression, it can be completely removed using a CsCl step gradient (22), since the DNA is much heavier than the virus particle. We previously observed that a DNA molecule in the viral stock can be 'transfected' into infected cells (35). (iv) The tissue-/cancer-specific promoter is inserted at the E4 position and is located farthest from the enhancer of a potent and non-specific EF1α promoter inserted at the E1-insertion site present near the left end of the genome. Therefore, the enhancer effect of the EF1α promoter on the specific AFP promoter is minimized. (v) A poly(A) sequence in front of the specific AFP promoter in the switch unit (Figure 1, upper right) suppresses non-specific transcription through a cryptic promoter present upstream of the specific promoter (3). And finally (vi) another poly(A) sequence in front of the *lox*P-cDNA in the target unit (Figure 1, upper left, and Figure 3) efficiently reduce the non-specific expression of cDNA probably caused by upstream cryptic promoters. We cannot argue whether the double-unit system is better in selectivity and less 'leaky' than the

authentic AFP promoter used here; for such conclusion further studies are needed using a more sensitive reporter system. However, because of these reasons above, the double-unit vector copes with high-level expression and strict specificity.

The total length of the AdV genome used in this method should not be more than ~38 kb (28). This means that the maximum length of a specific promoter plus target cDNA in this vector system with *Bsu*36I-*Blp*I E3 deletion (see below) should be 3.9 kb when the 14EF1α promoter is used and 4.5 kb when the 0.8-kb CMV promoter is used. This limitation of the length does not cause a problem in most cases. For example, because the AFP promoter used here is 2.2 kb in length, herpes thymidine-kinase cDNA (1.2 kb) or luciferase cDNA (1.7 kb) can be inserted; in fact, we constructed a double-unit AdV containing both AFP promoter and the thymidine-kinase gene, and animal experiments examining suicide-gene therapy are presently underway.

The problem of the genome length limitation can be solved using an AdV with a larger deletion in the E3 and E4 region. In the present study, AxLR14EL-AC and AxLR16EL-AC carried a *Bsu*36I-*Blp*I E3 deletion of 2433 bp, corresponding to 28 342–30 775-nt positions in the adenovirus type 5 map. The E3 region was 555-bp shorter than the *Xba*I-*Xba*I deleted E3 (21,27) and 252-bp longer than the *Bgl*II-*Bgl*II deleted E3 (36,37). We avoided using the *Bgl*II-*Bgl*II deleted E3 because L4 mRNAs of this AdV miss the L4 poly(A) sequences but use E3 poly(A) sequences, while *Xba*I-*Xba*I and *Bsu*36I-*Bpl*I deleted E3 both use the authentic L4 poly(A) sequences. Since an even shorter E3 region (27 865–30 995 nt) (36,38) and an E4 deletion (32 825–35 640 nt) (38) have been reported, the 3.9-kb limitation of the total lengths of a specific promoter plus cDNA when using AxLR14EL-AC could be enlarged up to 7.4 kb, if these vector constructs could be used.

Interestingly, Huyn *et al.* (39) recently reported an apparently related but different AdV system where, as the switch unit, a cancer-specific promoter produces a Gal4-VP16 fusion protein and, as the target unit, a Gal4-binding domain plus a CMV minimal promoter is used. They claimed that their method might be useful for visualizing cancer metastasis. A comparison of our vector with theirs would be difficult because they confirmed promoter specificity only in transfection experiments and because a cancer-specific promoter different from ours was used. Since our vector was developed with the goals of not only achieving a high expression level, but also of achieving a very low background in applications and ensuring the safety of gene therapy, the purposes of these studies are clearly different.

The method described here was totally different from that used for cancer-specific, replication-competent AdVs (for reviews, see references 40,41) in the field of cancer gene therapy. Although these AdVs use cancer-specific promoters, the adenoviruses replicate in the target cells and produce damaging effects through adenovirus gene expression in the target and surrounding cells. Although it has been reported that replication of E1-dereted AdV can be detected by Southern hybridization technique in HeLa cells at a higher MOI (42) and in other certain cells two weeks after infection (43), the replication level seemed too low to influence on the results described here.

The AdV system described here is probably useful for studying the function of a gene product in a specific tissue or organ. In any given tissue, several different sorts of cells are present: for example, neurons, glia cells and vascular endothelial cells are simultaneously present in neural tissue. Thus, this vector would be useful for expressing a gene selectively and efficiently in only one type of cell using a cell-specific promoter. This activity could enable novel, specific and effective therapies to be eveloped in the field of cancer gene therapy, and this strategy is now being tested. Furthermore, the application of this vector could be extended to include those where a high expression level and a rigid specificity are necessary.

The vector described here will be useful for many researchers using tissue/cancer-specific promoters. Plasmids suppressing Cre activity, cosmid cassettes for the construction of double-unit AdV containing *trc*-dnCreRY, and the 293 cell lines 293dnCreRY8 and 293shCreD13 are available from Riken Bioresource Bank (http://www.brc.riken.go.jp/) or in collaboration basis.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

ACKNOWLEDGMENTS

The authors thank Ms E. Kondo for her excellent secretarial assistance.

FUNDING

This work was supported by the Grant in Aid for Scientific Research on Priority Areas from Ministry of Education, Culture, Sports, Science and Technology, Japan (to I.S.). Funding for open access charge: Institute of Medical Science, University of Tokyo.

Conflict of interest statement. None declared.

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Oncology

Oncology 2011;81(suppl 1):11–17
DOI: 10.1159/000333253

Published online: December 22, 2011

Hepatocarcinogenesis in Hepatitis C: HCV Shrewdly Exacerbates Oxidative Stress by Modulating both Production and Scavenging of Reactive Oxygen Species

Hidetake Fujinaga Takeya Tsutsumi Hiroshi Yotsuyanagi Kyoji Moriya
Kazuhiko Koike

Department of Internal Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Key Words
Hepatitis C · Hepatocellular carcinoma · Oxidative stress · Transgenic mouse · Core protein

Abstract
Persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). One of the characteristics of HCV infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, in which HCC develops late in life after the preneoplastic steatosis stage, the core protein of HCV was shown to induce the overproduction of reactive oxygen species (ROS) in the liver. In excessive generation of ROS, HCV affects the steady-state levels of a mitochondrial protein chaperone, i.e. prohibitin, leading to an impaired function of the mitochondrial respiratory chain with the overproduction of ROS. Insulin resistance and hepatic steatosis, which frequently accompany HCV infection, exacerbate ROS production. On the other hand, HCV compromises some of the antioxidant systems, including heme oxygenase-1 and NADH dehydrogenase quinone 1, resulting in the provocation of oxidative stress, together with ROS overproduction, in the liver with HCV infection. Thus, HCV infection not only induces ROS but also hampers the antioxidant system in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. Combination with the other activated pathway, including an alteration in the intracellular signaling cascade of MAP kinase, along with HCV-associated disturbances in lipid and glucose metabolism would lead to the unusual mode of hepatocarcinogenesis, i.e. very frequent and multicentric development of HCC, in persistent HCV infection.

Introduction

Approximately 200 million people are infected with hepatitis C virus (HCV) worldwide. More than two thirds of those with acute HCV infection suffer from persistent infection causing active or inactive chronic hepatitis, and approximately 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime. Once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated and can explain at least part of the clinical progression of the disease. The patho-

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Kazuhiko Koike, MD, PhD
Department of Gastroenterology, Graduate School of Medicine
The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)
Tel. +81 3 5800 8800, E-Mail kkoike-tky@umin.ac.jp

genesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection; the role of viral proteins in the pathogenesis has also been reported [2]. Of the proteins constituting HCV, the core protein in particular has various functions with respect to host cells and is closely related to oxidative stress. In this article, the relationship between HCV infection and oxidative stress is analyzed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease is discussed.

HCV Infection and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet. Inflammation induced by an immune response to HCV should be considered, of course, in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in HCV infection with such a high incidence (90% in 15 years) or in a multicentric fashion? The other role of HCV would have to be weighed against a rare occurrence of HCC, even after the development of cirrhosis, in patients with autoimmune hepatitis in which severe inflammation in the liver persists. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C in vivo using transgenic mouse technology.

Transgenic Mouse Model for HCV-Related HCC

One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. For this purpose, several lines of mice have been established which are transgenic for the HCV cDNA. We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 4]. Four different kinds of transgenic mouse lines are established, and they carry the core gene, envelope genes, the entire nonstructural (NS) genes, or the NS5A gene, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [4]. The envelope gene transgenic mice did not develop HCC despite high expression levels of both E1 and E2 proteins [5], and the transgenic mice carrying the entire NS or NS5A gene developed no HCC.

Early in life, core gene transgenic mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [6]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. It is important to note that no significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the structural genes including the core gene [4, 7, 8]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Augmentation of Oxidative Stress in Hepatitis C

There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei [4]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in the core gene transgenic mouse model in the absence of inflammation in the liver [4]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage [2].

Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [2, 9]. Reactive

oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [10, 11]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [9].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, but little is known regarding the mechanism of increased oxidative stress in HCV infection. Hence, it is important to understand the mechanism of oxidative stress augmentation, in terms of both generation and scavenging of ROS, which may allow us to develop new tools of therapies for chronic hepatitis C.

Oxidative Stress and the Liver

Oxidative Stress and Reactive Oxygen

The main source of ROS in hepatocytes is the mitochondria. Outside of hepatocytes, ROS also originate from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. A large percentage of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS). Hepatocytes contain many mitochondria and therefore have a high ROS production. Generated ROS are very unstable and highly reactive and attack biomolecules such as DNA, lipids, and proteins. The liver not only produces much ROS but is also the center of the antioxidative effect in the form of protein synthesis. Oxidative stress refers to the oxidation-reaction-dominant state of the living body induced by an imbalance between the oxidation reaction caused by ROS and the antioxidation reaction. Main ROS include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$). ROS are mainly produced from $\cdot O_2^-$ and converted into stable H_2O_2 through a dismutation reaction. H_2O_2 is converted into highly reactive $HO\cdot$ in the presence of a transition metal.

The Antioxidant System and Oxidative Stress Markers

Antioxidants include glutathione (GSH), thioredoxin (TRX), vitamin E, vitamin C, and β-carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), GSH peroxidase, heme oxygenase (HO)-1, and catalase. SOD is induced by oxidative stress and dismutates $\cdot O_2^-$ to H_2O_2 and oxygen. Catalase in peroxisomes also decomposes H_2O_2 to water and oxygen. TRX is also a protein induced by oxidative stress and is reduced via S-S binding of the substrate protein by two SH groups in TRX and acts on the H_2O_2 elimination system via peroxiredoxins. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities.

ROS cause various forms of cellular damage. 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are the peroxidation reaction products of lipids, and 8-hydroxydeoxyguanosine (8-OHdG) is the product of DNA base modification. These products serve as oxidative stress markers.

The Origin of ROS Production in HCV Infection

Then, where is the place for oxidative stress overproduction in the liver of hepatitis C patients? The core protein is mostly localized to the endoplasmic reticulum, but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice [12]. In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core gene transgenic mice. Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of ROS. In livers of transgenic mice harboring the core gene, increased ROS production has been observed [2]. A recent study found, via proteomic profiling of biopsy specimens, that impairment of key mitochondrial processes including fatty acid oxidation and oxidative phosphorylation and of the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis [13]. Therefore, it is probable that the HCV core protein affects mitochondrial functions since such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. We performed a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and

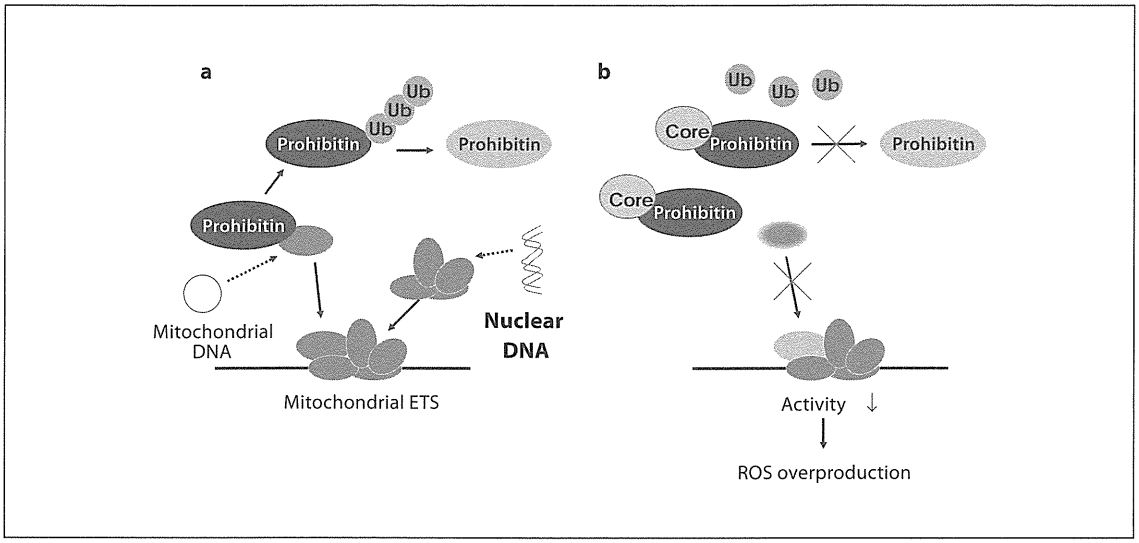

Fig. 1. The HCV core protein binds prohibitin and impairs its chaperone function leading to ROS overproduction. **a** Mitochondrial proteins consist of nuclear DNA-encoded proteins as well as mitochondrial DNA-encoded ones. Prohibitin acts as a protein chaperone for the mitochondrial proteins that are encoded by mitochondrial DNA by stabilizing newly synthesized mitochondrial translation products through direct interaction. **b** The HCV core interacts with prohibitin, disturbing its molecular chaperone function, and leads to a decrease in the activity of ETS complex IV, COX. Subunit II of COX is encoded by the mitochondrial DNA, while other subunits are encoded by the nuclear DNA. This is a new mechanism for oxidative stress overproduction in viral infection in that HCV induces mitochondrial ETS dysfunction by inhibiting chaperone function. Ub = Ubiquitin.

identified several proteins of different expressions when compared with control HepG2 cells. Among upregulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperone, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity.

Prohibitin, a mitochondrial protein chaperone, was identified as an upregulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell cycle progression and cellular proliferation by attenuating DNA synthesis [14]. It is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells. Mitochondrial prohibitin acts as a protein chaperone by stabilizing newly synthesized mitochondrial translation products through direct interaction [15]. We examined the interaction between prohibitin and the mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX) [15] (fig. 1). Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. In respect to the complex I function, we previously found a decreased complex I activity in core-expressing cells. Other groups have also shown that complex I activity is decreased in cultured cells [16]. Based on these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of prohibitin function has been shown to result in an increased production of ROS [17], a phenomenon observed in the core-expressing cells used in this study as well as in the liver of core-gene transgenic mice [2]. Interestingly, Shelly Lu et al. [18] recently reported that the liver-specific deletion of prohibitin resulted in morphological abnormality and HCC.

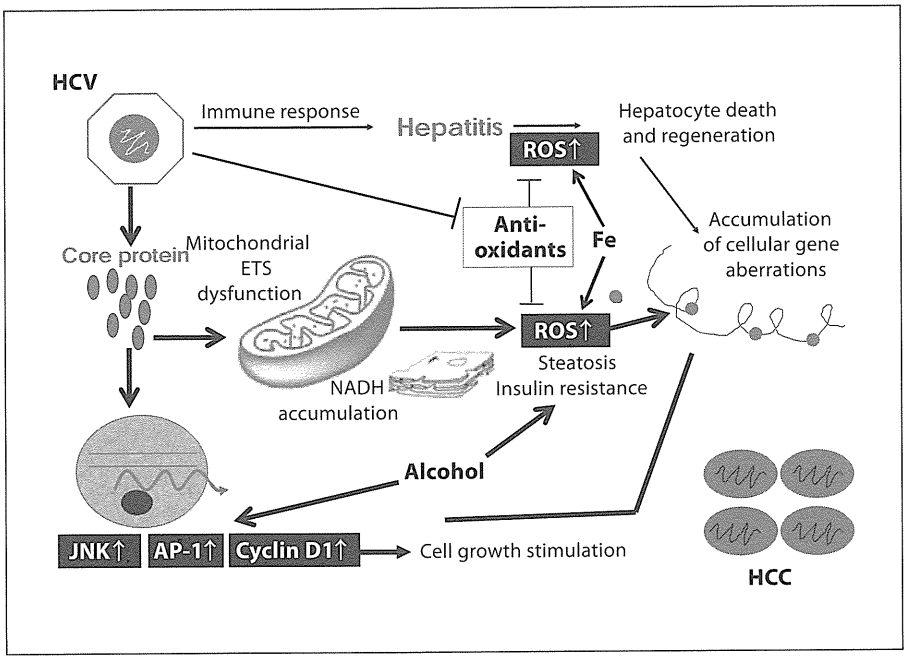

Fig. 2. Molecular pathogenesis of HCC development in HCV infection. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus itself contributes to hepatocarcinogenesis via two pathways. In one pathway, the core protein acts on the function of the mitochondrial ETS, leading to the overproduction of oxidative stress. The core protein also compromises some antioxidants and exacerbates ROS generation. Fe accumulation is an aggravating factor. The presence of steatosis and insulin resistance augments oxidative stress production. The other pathway is the modulation of cellular gene expression and signal transduction including the JNK pathway, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of HCC in HCV infection.

This is a new mechanism for ROS overproduction in viral infection in that HCV induces mitochondrial dysfunction through the inhibition of chaperone function in the mitochondria [19].

HCV Compromises the Antioxidant System

As discussed above, chronic hepatitis C is characterized by its prominent augmentation of oxidative stress. Related to this, iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [2, 9]. Iron is accumulated in the liver of HCV core gene transgenic mice [20]. The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates the observation in chronic hepatitis C patients [9, 10]. Then, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, some of the key antioxidant enzymes, including HO-1 and NADH dehydrogenase quinone 1 (NDQ-1), were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or non-iron-overloaded core gene transgenic mice. The attenuation of iron-induced augmentation of HO-1 was also confirmed in HepG2 cells expressing the core protein. HO-1 catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin, which is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities [21]. In addition, HO-1 has been also suggested to be a central antioxidant in conditions of GSH depletion [22]. Thus, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly in the case of iron overload. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection.

Thus, HCV infection not only induces ROS but also hampers antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis.

Conclusion

Pathways other than oxidative stress provocation in HCV-related hepatocarcinogenesis are alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [23]. The mitogen-activated pro-

tein kinase (MAPK) cascade, which is involved in numerous cellular events including cell proliferation, is also activated in the liver of the core gene transgenic mouse model. In the liver prior to HCC development, only the c-Jun N-terminal kinase (JNK) route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [23, 24]. Far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes. The combination of these pathways that are activated in HCV infection, i.e. ROS overproduction, attenuation of antioxidants, cell growth stimulation via MAPK activation, metabolic disturbances such as hepatic steatosis, and insulin resistance [25], which are all induced by HCV itself, would contribute to hepatocarcinogenesis, together with moderate but long-lasting inflammation in chronic hepatitis C (fig. 2).

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations [26]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis. On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute. The overall effect achieved by expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events which occur in HCV carriers. It no longer seem so difficult to determine why HCC develops in persistent HCV infection with an outstandingly high incidence. Our theory may also give an account of the multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Science, Sports and Culture of Japan; Health Sciences Research Grants of The Ministry of Health, Labour and Welfare (Research on Hepatitis).

Disclosure Statement

The authors have nothing to disclose.

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Original Article

Chronic hepatitis C in patients co-infected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

Hiroshi Yotsuyanagi,[1] Yoshimi Kikuchi,[2] Kunihisa Tsukada,[1,2] Kyouji Nishida,[3] Michio Kato,[4] Hironori Sakai,[5] Junki Takamatsu,[6] Shuhei Hige,[7] Kazuaki Chayama,[8] Kyoji Moriya[1] and Kazuhiko Koike[1]

[1]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo,* [2]*AIDS Clinical Center, International Medical Center of Japan,* [3]*Department of Laboratory Medicine, Tokyo Medical University, Tokyo,* [4]*Department of Gastroenterology, Osaka National Hospital, Osaka,* [5]*Department of Gastroenterology, Kyushu National Hospital, Fukuoka,* [6]*Division of Transfusion Medicine, Nagoya University Hospital, Nagoya,* [7]*Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo and* [8]*Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*

Aim: A nationwide survey in Japan revealed that nearly one-fifth of human immunodeficiency virus (HIV)-positive patients are co-infected with hepatitis C virus (HCV). We conducted a study to further analyze the features of liver disease in HIV–HCV co-infected patients.

Methods: We analyzed 297 patients from eight hospitals belonging to the HIV/AIDS Network of Japan.

Results: HCV genotypes 1, 2, 3, 4 and mixed genotypes were detected in 55.2, 13.7, 18.9, 0.9 and 11.3% of patients, respectively, in contrast to the fact that only genotypes 1 and 2 are detected in HCV mono-infected patients in Japan. This is compatible with the transmission of HCV through imported blood products contaminated by HCV. Sixteen of 297 HIV–HCV co-infected patients had advanced liver disease accompanied by ascites, hepatic encephalopathy or hepatocellular carcinoma. The average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications. The progression speed of liver disease estimated from the changes in the levels of serum albumin, bilirubin, or platelet was slower in patients who achieved sustained virological response with interferon treatment than in those who did not receive it. The overall sustained virological response rate to interferon treatment was 43.3%.

Conclusions: Our findings suggest that liver disease is more advanced in HIV–HCV co-infected patients than in HCV mono-infected patients, and interferon treatment may retard the progression of liver disease in such patients.

Key words: acquired immunodeficiency syndrome, chronic liver disease, genotype, interferon therapy

INTRODUCTION

THE PROGNOSIS OF human immunodeficiency virus (HIV) infection has markedly improved since the introduction of hyperactive anti-retroviral therapy (HAART).[1,2] Opportunistic infection has been prevented or properly managed, resulting in lower mortality rates. Liver disease, in particular related to hepatitis C virus (HCV) infection, has now become the main cause of mortality among HIV-infected patients on HAART in Western countries.[3,4] A national survey among Japanese HIV-infected patients with coagulation disorders has shown that the mortality rate related to HCV-related liver disease after 1997 was twofold that before 1997.[5] In Japan, therefore, HCV infection may also be a major cause of death in HIV–HCV co-infected patients. However, there has been no extensive analysis of liver disease in HIV–HCV co-infected patients in Japan.

Correspondence: Professor Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-tky@umin.ac.jp
Received 20 January 2009; revised 9 February 2009; accepted 10 February 2009.

Interferon (IFN) treatment in combination with ribavirin administration, which is now the first choice for HCV mono-infected patients,[6] is also a standard treatment for chronic hepatitis in HIV–HCV co-infected patients. Eradication of HCV is assumed to improve liver function, and normalization of serum aminotransferase (ALT) levels by IFN treatment may retard the progression of liver disease in HIV–HCV co-infected patients, even if they are on HAART. However, in general, the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients.[7] The effects of IFN treatment on liver function and prognosis in HIV–HCV co-infected patients in Japan are yet undefined.

In 2004, we conducted a nationwide survey to determine the prevalence of HCV infection in HIV-infected patients by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan, which revealed that 935 (19.2%) of 4877 HIV-positive patients were also positive for anti-HCV antibody.[8] In this study, we analyzed the progression of liver diseases and the impact of IFN treatment on the parameters of liver function in HIV–HCV co-infected patients in a multicenter retrospective study.

METHODS

Registry of patients with HIV–HCV co-infection

THE QUESTIONNAIRE REGARDING the current state of HIV–HCV co-infection was sent to the 366 hospitals in the HIV/AIDS Network of Japan in 2004, sponsored by the Japanese Ministry of Health, Labour and Welfare. One hundred seventy-six hospitals (48.1%) responded. The results, already published,[8] showed that HIV–HCV co-infected patients are concentrated in particular hospitals in big cities around Japan. Among these hospitals, we chose three hospitals in the Tokyo metropolitan area, and one each in the Hokkaido, Chubu, Osaka, Chugoku and Kyushu areas. These eight hospitals belong to the HIV/AIDS Network and had more HIV–HCV co-infected patients than other hospitals.

In the study, the following information was obtained from the hospitals regarding each HIV–HCV co-infected patient who visited the hospitals at least once between January and December in 2004: (1) age and sex of HIV-positive patients with anti-HCV; (2) possible transmission routes of HIV; (3) history of habitual alcohol intake; (4) date of the first and last visits; (5) counts of white blood cells, CD4-positive lymphocytes and platelets at the first and last visits; (6) levels of serum albumin and bilirubin at the first and last visits; (7) levels of HIV-RNA and HCV-RNA at the first and last visits; (8) history of IFN treatment with or without ribavirin; (9) history of HAART; and (10) history of jaundice, ascites, hepatic encephalopathy and hepatocellular carcinoma (HCC). The study sheets were completed by the physicians in charge and sent to the Department of Internal Medicine, University of Tokyo.

Ethical issues

The protocol of the current survey was approved by the ethical committee of each institution, and written informed consent was obtained from each patient.

Statistical analysis

The collected data were analyzed using Mann–Whitney's *U*-test whenever appropriate. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Clinical backgrounds of registered patients

FROM THE EIGHT hospitals, 297 patients were registered. The number, age, sex, estimated transmission routes and history of habitual alcohol intake are shown in Table 1. Two hundred and ninety (97.6%) were male patients. The mean age of the patients was 37.9 ± 10.3.

HCV genotype was determined in 212 patients. One hundred seventeen (55.2%) patients were infected by genotype 1 HCV. Infection by genotypes 2, 3 or 4 HCV was found in 29 (13.7%), 40 (18.9%) and 2 (0.9%) patients, respectively. Twenty-four (11.3%) patients were infected by HCV of mixed genotypes. In the remaining 85 patients, the genotype was indeterminable or undetermined. The mean ages of patients infected by different HCV genotypes were similar (Table 1).

In 259 (87.2%) of 297 registered patients, HIV was most probably transmitted through the administration of blood products. Other transmission routes were sexual contacts among men who have sex with men (MSM) (4.0%), heterosexual contacts (3.0%) and intravenous drug use (IDU) (0.3%). Habitual alcohol consumption was noted in only one patient with genotype 1 HCV (0.6%).

Outcomes of IFN treatment in HIV–HCV co-infected patients

Serum HCV-RNA levels were available both at the first visit and registry to the study (i.e. the end of observa-

Table 1 Demography, transmission route and HCV genotypes in HIV–HCV co-infected patients

HCV genotype	Number (%)	HCV sub-genotypes	Viral load† (High : Low)	Age	Sex (Male : Female)	Transmission route				
						Transfusion	MSM	Hetero-sexual	IDU	Others
1	117 (55.2)	1a 31, 1b 43, 1a+1b 31, undetermined 12	31:11	38.3 ± 10.4	114:3	102	7	1	0	7
2	29 (13.7)	2a 16, 2b 11, undetermined 2	5:5	39.8 ± 9.5	29:0	24	1	1	0	3
3	40 (18.9)	3a 40	12:2	36.1 ± 8.9	40:0	38	0	0	0	2
4	2 (0.9)	4a 2	2:0	38.5 ± 2.1	2:0	2	0	0	0	0
Mixed	24 (11.3)	2a+3a 6, 1b+3a 3, others 15	11:0	38.7 ± 8.7	24:0	24	0	0	0	0
Others	85	Undetermined 85	6:1	36.2 ± 11.5	81:4	69	4	7	1	4
Total	297		67:19	37.9 ± 10.3	290:7	259 (87.2%)	12 (4.0%)	9 (3.0%)	1 (0.3%)	16 (5.5%)

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.
HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, intravenous drug users; MSM, men who have sex with men.

tion) in 158 patients. Of these 158, 60 patients (38.0%) received IFN treatment for HCV, and 35 of these 60 patients did it in combination with ribavirin. Those who did not complete the scheduled treatment were excluded from the current analysis.

As shown in Table 2, 26 (43.3%), 11 (18.4%) and 23 (38.3%) of the treated patients achieved sustained virological response (SVR), end-of-treatment virological response (ETR) and no virological response (NR), respectively. The SVR rate in patients with each genotype is shown in Table 2. The SVR rate in the patients who underwent IFN treatment in combination with ribavirin was 31.4% in total. The SVR rate in patients with each genotype who underwent IFN/ribavirin combination therapy is shown in Table 2.

All of the 26 patients who achieved SVR remained negative for serum HCV-RNA in the further follow-up periods. In contrast, none of the patients with ETR or NR became negative for serum HCV-RNA in the follow-up periods. In five patients who did not receive IFN treatment, HCV-RNA was negative at the end of the observation period, although it was positive at least twice before the registry. The profiles of the five patients are shown in Table 3.

Changes in liver function and associated complications (Table 4)

As mentioned above, the data on liver function and serum HCV-RNA positivity were available both at the first visit and registry (end of observation) in 158 of the 297 registered patients. The mean observation period was 9.5 ± 5.0 and 8.2 ± 8.2 years in the IFN-treated and IFN-untreated patients, respectively. Unfortunately, few, if any, patients underwent liver biopsy, because most HIV–HCV co-infected patients had coagulation disorders.

The annual change in the serum albumin concentration was +0.05 ± 0.42 g/dL in the IFN-treated patients, and −0.80 ± 0.82 g/dL in the non-IFN-treated patients. The annual change in the serum bilirubin concentration was +0.08 ± 0.38 mg/dL in the IFN-treated patients, while it was +0.15 ± 0.15 mg/dL in the non-IFN-treated patients. Among the IFN-treated patients, the serum bilirubin concentration decreased by 0.02 ± 0.08 mg/dL in the patients who achieved SVR, which was significantly larger than that in the non-IFN-treated patients at the end of the observation ($P < 0.05$). The annual changes in platelet counts were +0.06 ± 1.13 ($\times 10^4/\mu l$) in the IFN-treated patients and −0.94 ± 0.95 ($\times 10^4/\mu l$) in the non-IFN-treated patients. The change in platelet

Table 2 Virological response to interferon treatment in HIV–HCV co-infected patients

Genotype	Viral load (High : Low)†	Response			Total
		SVR	ETR	NR	
(a) Response to interferon treatment in total (with or without ribavirin)					
1	9:6	7 (33.3%)	1	13	21
2	5:3	4 (40.0%)	2	4	10
3	5:1	5 (62.5%)	1	2	8
4	1:0	0	1	0	1
Mixed	5:1	2 (33.3%)	3	1	6
Others	6:2	8 (57.1%)	3	3	14
Total	31:13	26 (43.4%)	11	23	60
(b) Response to ribavirin/interferon combination therapy including peginterferon					
1	8:2	2 (15.3%)	0	11	13
2	1:2	1 (25.0%)	0	3	4
3	4:1	4 (66.7%)	1	1	6
4	1:0	0	1	0	1
Mixed	4:1	1 (20.0%)	3	1	5
Others	3:0	3 (50.0%)	1	2	6
Total	21:6	11 (31.4%)	6	18	35

†Viral loads are available in only a subset of patients. High viral load: more than 1 Meq/mL by Branched DNA-probe assay or more than 100 KIU/mL by Amplicor monitor assay.
ETR, end of treatment virological response; NR, no virological response; SVR, sustained virological response.

counts in the patients who achieved SVR was significantly larger than that in the non-IFN-treated patients ($P < 0.05$, Table 4).

No symptoms of hepatic failure (ascites or hepatic encephalopathy) were observed in the 60 IFN-treated patients while they were observed in six of the 98 non-IFN-treated patients. HCC was found in one IFN-treated patient after SVR, while it was found in two non-IFN-treated patients (Table 4).

Impact of HAART on liver function and associated complications (Table 5)

Information on HAART was available in 292 patients. The mean observation periods were 8.4 ± 4.2 years in 234 patients on HAART, and 9.8 ± 6.0 years in 58 patients not on HAART. Changes in the levels of albumin, bilirubin or platelet were similar between the two groups (statistically not significant). The morbidities of hepatic decompensation symptoms (ascites and hepatic encephalopathy) and HCC were not significantly different between the two groups. In total, nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was 41.1 ± 14.0 years, which was much younger than that of HCV mono-infected patients with the same complications.[9]

DISCUSSION

IN THE CURRENT study, the features of liver disease in HIV–HCV co-infected patients in Japan were analyzed. The determination of HCV genotypes revealed that genotype 3 or 4, which is rarely seen in HCV mono-infected patients in Japan,[10] was found in a substantial fraction of HIV-infected patients. In addition, some of these patients were infected with HCV of mixed genotypes. These results are compatible with the fact that HCV is transmitted through imported blood products that were contaminated by HCV, as is the case with HIV infection.[11] Infection by HCV of mixed genotypes may reflect frequent administrations of blood products of different lots.

We evaluated the response rate to IFN treatment in HIV–HCV co-infected patients in Japan. Because the IFN treatment protocol varied between facilities, it was not easy to evaluate the effects of the treatments including IFN in this cohort. However, the regimen of ribavirin/IFN combination therapy was similar between the hospitals: the treatment period was 24 weeks in patients with HCV genotypes 2 and 3, and 48 weeks in those with HCV of other genotypes when either pegylated or standard IFN in combination with ribavirin was used.[12] Therefore, it may be possible to estimate the effect

Table 3 Clinical backgrounds of patients who spontaneously cleared HCV in HIV-infected patients

Patient no.	Age	Sex	Transmission route	Observation period (years)	HCV-RNA (KIU/mL)	HCV genotype	HIV-RNA ($\times 10^2$/mL)	WBC (/μL)	CD4+ T cells (/μL)	Platelets ($\times 10^4$/mL)	ALT (U/l)	HAART
1	33	M	Transfusion	8.8	290	ND	200 000	4500	5	26.3	21	Yes
2	31	M	MSM	2.3	Positive†	ND	13 000	5760	931	22.7	29	Yes
3	27	M	Transfusion	9.3	>850	3a	180 000	4000	51	10.1	84	Yes
4	53	M	Transfusion	4.5	Positive†	1a	20 000	4800	296	35.4	24	No
5	22	M	Transfusion	7.8	220	ND	990	5500	125	33.1	44	Yes

†Positive: HCV-RNA was positive by qualitative PCR, but was not quantitatively determined.
ALT, aminotransferase; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; ND, not determined; WBC, white blood cells.

Table 4 Changes in clinical parameters and IFN treatment in HIV–HCV co-infected patients

	Outcome of IFN treatment	Number	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	Ascites/ encephalopathy	HCC
IFN-treated patients		60	9.5 ± 5.0	0.05 ± 0.42	0.08 ± 0.38*	0.06 ± 1.13	0	1
	SVR	26	9.1 ± 4.4	0.13 ± 0.59	(−) 0.02 ± 0.08*	0.14 ± 0.76*	0	1
	ETR	11	14.6 ± 7.0	(−) 0.07 ± 0.14	0.51 ± 1.04	0.07 ± 1.50	0	0
	NR	23	7.4 ± 2.0	0.01 ± 0.30	0.09 ± 0.30	(−) 0.18 ± 0.32	0	0
Non-IFN-treated patients		98	8.2 ± 8.2	(−) 0.80 ± 0.82	0.15 ± 0.15	(−) 0.94 ± 0.95	6	2
All		158	8.7 ± 4.7	(−) 0.45 ± 2.93	0.13 ± 0.52	(−) 0.59 ± 3.78	6	3

*$P < 0.05$ versus patients without IFN treatment.
†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times 10^4$/μL)/observation period (years).
ETR, end of treatment virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; NR, no virological response; SVR, sustained virological response.

Table 5 Changes in clinical parameters and HAART in HIV–HCV co-infected patients

	Number	Age	Sex (M : F)	Observation period (years)	⊿Albumin†	⊿Bilirubin‡	⊿Platelet§	IFN	Ascites/ encephalopathy	HCC
HAART (+)	234	37.8 ± 10.4	227:7	8.4 ± 4.2	(–) 0.002 ± 0.18	0.13 ± 0.53	(–) 0.40 ± 3.71	143 (61.1%)	6	5
HAART (–)	58	38.1 ± 10.5	58:0	9.8 ± 6.0	(–) 0.14 ± 0.18	0.03 ± 0.25	(–) 1.40 ± 3.30	30 (51.7%)	3	2

†⊿Albumin: changes in albumin concentration (g/dL)/observation period (years).
‡⊿Bilirubin: changes in bilirubin concentration (mg/dL)/observation period (years).
§⊿Platelet: changes in platelet count ($\times 10^4$/[L)/observation period (years).
HAART, highly active anti-retroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

of ribavirin/IFN combination therapy in HIV–HCV co-infected patients in this study.

The response rate to ribavirin/IFN combination therapy was 31.4% in total, and 15.3% in patients with HCV genotype 1, which are comparable rates to those achieved in previous studies on HIV–HCV co-infected patients in Western countries.[7] The low response rate in HIV–HCV co-infected patients compared with HCV mono-infected patients[12] may be attributed to several factors: impaired immune response, high HCV loads and viral quasi-species caused by frequent chances of transmission. Of these, high viral loads may be essential, because Table 2 shows that patients with genotype 1 HCV achieved SVR even by IFN monotherapy if their viral loads were low. In the era of IFN monotherapy, patients with favorable conditions were treated first of all: pretreatment viral loads in patients who received IFN monotherapy were lower than those who received PEG-IFN–ribavirin combination therapy. This may be the reason why the efficacy of PEG-IFN–ribavirin combination therapy was lower than that with IFN monotherapy in this study.

The serum bilirubin concentrations and platelet counts were improved in the patients who achieved SVR by IFN treatment. Although the response rate to IFN treatment is lower in HIV–HCV co-infected patients than in HCV mono-infected patients, the overall benefit of IFN treatment on liver function may be similarly expected in the patients who achieved SVR. HAART showed no impact on the liver function in HIV–HCV co-infected patients. Improvement of liver function can be expected only in IFN-treated patients, although there is a possibility that only patients with preserved liver function were able to receive IFN treatment. Given that liver disease is the major life-threatening factor in HIV-infected patients, IFN treatment should be considered in the early stage of HIV–HCV co-infection.

It should be noted that nine patients had hepatic decompensation and seven had HCC, and the average age of such patients was much younger than that of HCV mono-infected patients with the same complications.[9] This finding is compatible with reports from Western countries showing a faster progression of fibrosis[13] and earlier development of HCC.[14] A possibly interesting finding is that five patients (approximately 3% of patients whose serum HCV-RNA level was serially determined) cleared HCV-RNA from the serum without IFN treatment. Previous reports showed that some HIV-infected patients could spontaneously clear HCV-RNA.[15–17] The clearance of HCV among patients with chronic HCV infection is rare, although it has been

reported in Japan.[18] Three of the five patients had high HCV loads and low $CD4^+$ T-lymphocyte counts, which are generally thought to be unfavorable for spontaneous HCV clearance. A difference in immune status of HIV-infected patients from HCV mono-infected patients may be involved in such an observation, although further studies are awaited.

In summary, our study demonstrated that approximately 20% of HIV-infected patients are co-infected with HCV. Some of the HIV–HCV co-infected patients had advanced liver disease such as ascites, encephalopathy or HCC at a younger age than HCV mono-infected patients, suggesting that the progression of liver disease may be more rapid in HIV–HCV co-infected patients than in HCV-mono-infected ones. Treatments with regimens including IFN, which may improve liver function and decrease liver-related death, should be considered in HIV–HCV co-infected patients.

ACKNOWLEDGMENTS

WE THANK MS Ogawa for her assistance in the questionnaire inquiry. This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan (AIDS Research).

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Proteomics Analysis of Mitochondrial Proteins Reveals Overexpression of a Mitochondrial Protein Chaperon, Prohibitin, in Cells Expressing Hepatitis C Virus Core Protein

Takeya Tsutsumi,[1] Mami Matsuda,[2] Hideki Aizaki,[2] Kyoji Moriya,[1] Hideyuki Miyoshi,[1] Hajime Fujie,[1] Yoshizumi Shintani,[1] Hiroshi Yotsuyanagi,[1] Tatsuo Miyamura,[2] Tetsuro Suzuki,[2] and Kazuhiko Koike[1]

The hepatitis C virus (HCV) core protein is involved in viral pathogenesis such as oxidative stress induction and lipid metabolism disturbance, and is primarily located in the cytoplasm and endoplasmic reticulum in association with lipid droplets as well as in the mitochondria. To clarify the impact of the core protein on mitochondria, we analyzed the expression pattern of mitochondrial proteins in core protein-expressing cells by two-dimensional polyacrylamide gel electrophoresis. Several proteins related to the mitochondrial respiratory chain or protein chaperons were identified by mass spectrometry. Among the identified proteins with consistently different expressions, prohibitin, a mitochondrial protein chaperon, was up-regulated not only in core-expressing cells but also in full-genomic replicon cells and livers of core-gene transgenic mice. The stability of prohibitin was increased through interaction with the core protein. Further analysis demonstrated that interaction of prohibitin with mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX) was disturbed by the core protein, resulting in a significant decrease in COX activity. *Conclusion:* The HCV core protein affects the steady-state levels of a subset of mitochondrial proteins including prohibitin, which may lead to an impaired function of the mitochondrial respiratory chain with the overproduction of oxidative stress. (HEPATOLOGY 2009;50:378-386.)

The hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which often leads to cirrhosis and, eventually, to the development of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis in HCV infection is not yet fully elucidated. The HCV core protein forms the viral nucleocapsid protein and has various properties that modulate cellular processes in numerous ways. The core protein binds to cellular proteins, suppresses or enhances apoptosis, and modulates the transcription of some host genes.[1] In addition, transgenic mice expressing the core protein develop HCC,[2-4] indicating a direct contribution of the core protein to the pathogenesis of hepatitis C.

The core protein is mostly localized to the endoplasmic reticulum (ER), but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice.[2,5,6] In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core-gene transgenic mice.[2-4] Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of reactive oxygen species (ROS). In livers of transgenic

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; COX, cytochrome c oxidase; ER, endoplasmic reticulum; Ero1, ER protein endoplasmic oxidoreduction-1; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSP, heat shock protein; IFN, interferon; MnSOD, manganese superoxide dismutase; NS, nonstructural; OST48, oligosaccharyltransferases-48; PDH, pyruvate dehydrogenase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; TFA, trifluoroacetic acid.

From the [1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo; [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Received June 17, 2008; accepted March 20, 2009.

Supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan (Research on Hepatitis), from the Ministry of Education, Culture, Sports, Science and Technology (Priority Area), from The Sankyo Foundation of Life Science, and from The Charitable Trust Araki Memorial Promotion Fund. T. T. is an awardee of the Research Resident Fellowship from the Viral Hepatitis Research Foundation of Japan.

Address reprint requests to: Kazuhiko Koike, M.D., Ph.D., Department of Gastroenterology, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kkoike-tky@umin.ac.jp; fax: (81) 3-5800-8799

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22998

Potential conflict of interest: Nothing to report.

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