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Review

Oxidative stress and hepatitis C viral infection

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Abstract

The involvement of oxidative stress in the pathogenesis of hepatitis and hepatocellular carcinoma has been strongly suggested. Oxidative stress is produced by inflammatory processes that occur in hepatitis via immunological mechanisms. In addition, in hepatitis C virus (HCV) infectious disease, some role has been assigned to viral proteins in the induction of oxidative stress. In the presence of hepatic steatosis, insulin resistance and increased levels of some cytokines, all of which are also induced by viral protein expression, oxidative stress is enhanced in HCV infection. In this sense, the role of oxidative stress in the progression of chronic hepatitis and hepatocarcinogenesis is greater in hepatitis C than in other types of hepatitis such as hepatitis B or autoimmune hepatitis. The additive effects of oxidative stress caused by the inflammatory process and that induced by HCV proteins may, furthermore, exert synergistic effects with alterations in intracellular signaling systems such as mitogen-activated protein kinases (MAPK), which are also induced by HCV proteins. These synergistic effects may be responsible for rare characteristics, that is, the high incidence and multicentric nature of hepatocarcinogenesis in HCV infection.

Keywords: Oxidative stress; Hepatitis C virus; Hepatocarcinogenesis; Lipid peroxidation; Steatosis; Insulin resistance

Contents

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1. Introduction

There are approximately 200 million people infected with hepatitis C virus (HCV) worldwide, of which about 1.8 million are in Japan. It is one of the most serious causes of liver disease. It was reported that approximately 70% of those with HCV infection suffer from persistent infection, causing active or inactive chronic hepatitis and that about 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 5–7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated recently and it has become possible to explain at least part of the clinical progression of the disease. The pathogenesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection, but the role of viral proteins in the pathogenesis was also reported [2–4]. Of proteins constituting HCV, the core protein, in particular, has various functions with respect to host cells [5] and is closely related to oxidative stress. In this overview, the relationship between HCV infection and oxidative stress is reviewed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease will be discussed.

2. Oxidative stress, reactive oxygen, and the liver

2.1. Oxidative stress and reactive oxygen

The main source of reactive oxygen species (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. Several percent of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS, Fig. 1). 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 anti-oxidative 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 anti-oxidation reaction. Main ROS include superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$). ROS are mainly produced from $^{\bullet}O_2^-$ and converted into stable H_2O_2 through dismutation reaction. H_2O_2 is converted into highly reactive $HO^{\bullet}$ in the presence of a transition metal.

2.2. Antioxidation 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), glutathione peroxidase (GPx), and catalase. SOD is induced by oxidative stress and dismutates $^{\bullet}O_2^-$ to H_2O_2 and oxygen. GSH is a compound belonging to the SH group and is highly abundant in the living body, and the SH group provides electrons to free radicals to stabilize the radicals. GSH exists in a reduced form in cells. Because it is converted into dimeric oxidized glutathione (GSSG) and becomes stable after donating electrons, GSSG prevents free radicals from continuously scrambling for electrons. GPx decomposes H_2O_2 into water

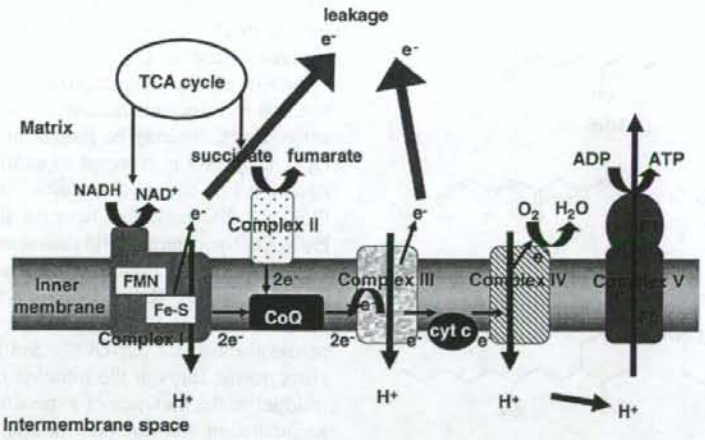

Fig. 1. The electron transfer system (ETS) of the mitochondrion. Most of the oxygen, consumed by mammalian cells, is converted to water via the mitochondrial ETS. However, up to 5% of the electrons entering the mitochondrial ETS can become uncoupled and singly leak out onto oxygen to form superoxide. Therefore, if there is impairment in the mitochondrial ETS function, it can be a cause of the overproduction of reactive oxygen species (ROS). TCA, tricarboxylic acid; NADH, nicotinamide adenine dinucleotide phosphate; FMN, flavine mononucleotide; CoQ, coenzyme Q; cyt c, cytochrome c.

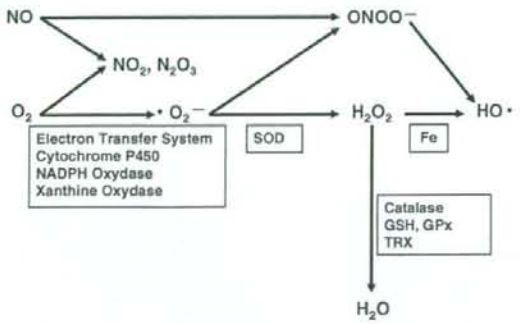

Fig. 2. Generation and scavenging of oxidative stress. SOD, super-oxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; TRX, thioredoxin.

and oxygen with GSH as an electron donor and reduces lipid peroxide to become neutralized. GSSG is converted back to GSH when glutathione reductase transfers an electron from NADPH to GSSG. 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 the S–S binding of the substrate protein by two SH groups in TRX and acts on the H_2O_2 elimination system via peroxiredoxins (Fig. 2).

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 (Fig. 3). These products serve as oxidative stress markers.

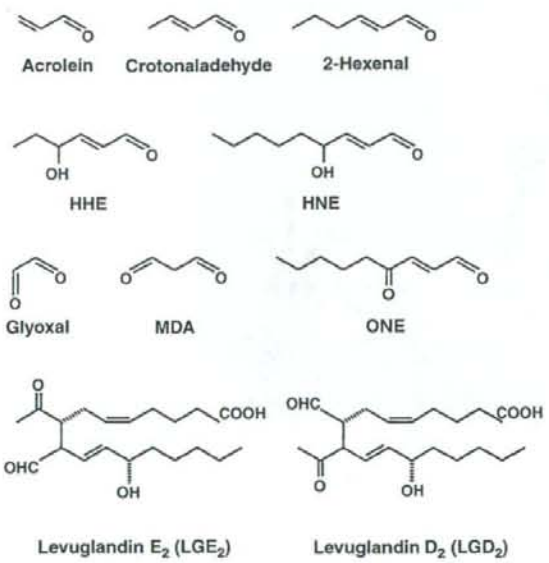

Fig. 3. Representative aldehyde species generated via lipid peroxidation reaction.

3. Viral infection and oxidative stress

3.1. ROS production associated with viral infection

Upon viral infection, ROS are produced by NADPH oxidase and xanthine oxidase in neutrophils and macrophages. In particular, NS3, one of the non-structural proteins of HCV, was reported to induce ROS production by NADPH oxidase in neutrophils [6]. Furthermore, in viral hepatitis, ROS are also produced in hepatocytes through the release of inflammatory cytokines such as TNF-α and IL-1β from inflammatory cells. Increased hepatic or serum 8-OHdG, HNE and MDA levels are observed in chronic hepatitis C, indicating an increase in ROS production [7–13]. Findings that indicate an increase in the activity of the ROS elimination system including decreased hepatic and blood GSH levels, an increased GSSG/GSH + GSSG ratio, and an increased serum TRX level have been reported [13–16]. The findings of markedly decreased HNE level following viral eradication with interferon [12] and decreased serum ALT and TRX levels following the administration of vitamin E, an antioxidant [17], also demonstrated that oxidative stress plays an important role in chronic hepatitis C.

3.2. Nitric oxide production associated with viral infection

In the presence of an inflammation, inducible nitric oxide synthase (iNOS) is induced in macrophages and hepatocytes by TNF-α and IFN-γ [18–20]. Other investigators reported that protein kinase (PKR) activated by double-stranded RNA formed during virus reproduction in turn activates the transcripts of NF-κB and IRF-1 to induce iNOS [21]. In the case of HCV, it was reported that its constituent proteins (E2 and non-structural (NS) protein 5A) inhibit PKR activity [22,23], but iNOS induction by viral RNA via PKR is also suspected. Indeed, iNOS synthesis correlates with intrahepatic viral load in chronic hepatitis C [24].

NO is generally synthesized as a non-specific defense reaction to infectious diseases; however, in viral infection, antiviral activity may be present or absent in various viral types [20]. NO is reported to exhibit no antiviral activity against a tick-borne encephalitis virus (TBE-V), flavivirus [25], and NO may also have no antiviral activity against HCV. On the contrary, NO causes cellular damage upon its reaction to O_2 or simultaneously produced O_2^- (reactive nitrogen species, RNS). Upon reaction to O_2^-, in particular, NO acts as a strong oxidant with the generation of peroxynitrous acid ($ONOO^-$), and $ONOO^-$ also produces nitrotyrosine through the nitration of aromatic amino acid residues in the presence of a transition metal. Nitrotyrosine accumulation was observed in correlation to inflammation severity in chronic hepatitis C tissue [26]; suggesting that the production of both NO and ROS increased. ROS and RNS are produced as defense factors for biological viral clearance, but these factors also have cytotoxic effects that

are assumed to contribute to the exacerbation of the disease state.

4. Oxidative stress caused by viral protein

The HCV genome comprises the genes of four structural proteins and six non-structural proteins (Fig. 4), and it has been reported that at least two viral proteins cause oxidative stress in cells. The core protein, a structural protein, was found to have various actions, including the induction of oxidative stress and accumulation of lipids, in experimental studies using cultured cells and transgenic mice [2,27]. Experiments using mice transgenic for the core gene showed an increased ROS production, an increased intrahepatic catalase activity, a decreased intrahepatic GSH level and a decreased GSH/GSH – GSSG ratio indicating an antioxidation effect inhibition, although there was no increase in serum ALT level nor a histological finding of hepatitis. Increased levels of intrahepatic peroxide lipids in the core gene transgenic mice with aging as compared with those in the control mice also indicate increased oxidative stress. As a mechanism underlying oxidative stress induction by the core protein, mitochondrial damage is considered. Morphological abnormalities of the mitochondria were observed in the core gene transgenic mouse liver [2], and an increased ROS production caused by damage of the mitochondrial electron transport system was noted in core-protein-expressing cells [27]. Mitochondrial DNA, which has no protective proteins such as histone, is susceptible to damage by ROS [28,29]. Mitochondrial DNA in the core gene transgenic mice showed damage as early as 3-months old. This mitochondrial damage disrupts the synthesis of proteins constituting the electron transport system complex and could also increase oxidative stress caused by damage of the electron transport system.

A study using a cell culture system demonstrated that nonstructural protein 5A (NS5A) also causes oxidative stress. NS5A induces endoplasmic reticulum calcium release via endoplasmic reticulum stress, and this leads to an increased ROS production in the mitochondria [4]. Although the effect of NS5A has not been confirmed yet by other study groups, HCV has the direct action of increasing intracellular ROS production via its proteins, separate from oxidative stress induction by inflammation caused by viral infection. A report that oxidative stress was also observed in HCV carriers with a normal ALT level [13] indicates that it is caused by a direct oxidative stress induction without being mediating inflammatory reactions.

5. Relationship of HCV infection with insulin resistance

The relationship of HCV infection with insulin resistance and type 2 diabetes has been suggested epidemiologically [30–32]. Insulin resistance was also observed in core gene transgenic mice before the onset of hepatic steatosis [33]. A disrupted tyrosine phosphorylation of the insulin receptor substrate (IRS-1) was observed in the liver of these transgenic mice. The analysis of hepatic tissues in patients with chronic hepatitis C not complicated by diabetes showed that insulin receptor and IRS-1 expression levels are elevated in patients with HCV infection, whereas the tyrosine phosphorylation of IRS-1 induced by insulin is inhibited. An excessive oxidative stress may be another potential cause of this insulin resistance. Oxidative stress indirectly blocks the phosphorylation of tyrosine residues of insulin receptors and IRS-1 and inhibits insulin signaling [34].

These reported results thus indicate an insulin signaling disorder in the liver infected with HCV [35]. There has been no report to date directly proving that hepatic insulin signaling disorder in patients with HCV infection is attributable to oxidative stress. However, because diabetes, which is the state of having abnormally high blood sugar levels that cannot be self-regulated by individual organisms, also induces oxidative stress [34], the close relationship between insulin resistance or diabetes and oxidative stress as the cause and the

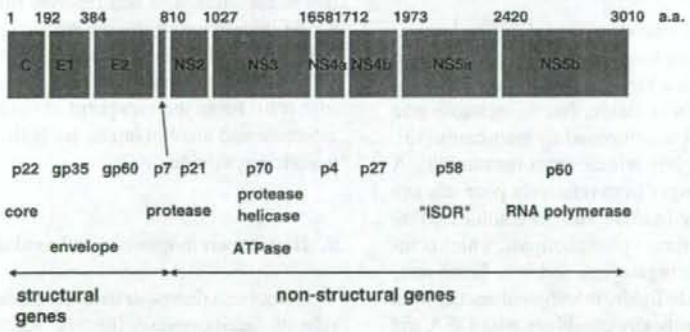

Fig. 4. Structure of hepatitis C virus genome. The genome of HCV consists of two parts, structural and non-structural regions. The former comprises the core and envelope regions, and the latter consists of NS2 to NS5a, which regions chiefly code enzymes necessary for viral replication. NS, non-structural; ISDR, interferon sensitivity-determining region.

result, respectively, is a very interesting issue to investigate in the future.

6. Relationship of HCV infection with hepatic steatosis

Hepatic steatosis is frequently observed in patients with HCV infection. The relationships of HCV infection with intrahepatic viral loads and core protein levels, different prevalence of hepatic steatosis by viral genotype [higher incidence for genotype 3a), and improved steatosis following viral eradication were reported [36–38]. It is presumed from these reports that HCV itself causes hepatic steatosis. A similar hepatic steatosis caused even by the core protein alone was observed in a study using an expression system in cultured cells and transgenic mice, and it was thus suggested that the core protein plays a significant role in hepatic steatosis as the direct action of HCV [39,40]. Hyperinsulinemia induced by insulin resistance mentioned above causes the overloading of the liver with fatty acids from fat cells, and mitochondrial damage inhibits the β-oxidation of fatty acids [41]. Furthermore, the core protein was reported to inhibit microsomal triglyceride transfer protein (MTP) activity that is required when neutral fat is released as very low-density liproproteins (VLDLs) [42]. All these actions could cause hepatic steatosis. In the liver of non-alcoholic steatohepatitis (NASH) patients, it was reported that β-oxidation in the mitochondria and peroxisomes or the metabolism of fatty acids by cytochrome P450 2E1 (CYP2E1) in microsomes is promoted under an excessive load of fatty acids, resulting in ROS production [43,44]. In HCV infection as well, intrahepatic fat accumulation possibly increases ROS production as in NASH. Because hepatic steatosis in chronic hepatitis C was reported to be a factor for disease progression [45–47], increased oxidative stress associated with hepatic steatosis is presumably involved in disease progression.

7. Iron and reactive oxygen

The iron content in the liver and spleen is high, and transition metals facilitate electron transfer and play an important role in the production of free radicals. Iron in combination with transferrin and ferritin is stable, but an unstable iron ion is freed when ferritin is decomposed by lysosomes [48]. ROS additionally promote iron release from ferritin [49]. A free iron ion catalyzes changes from relatively poor reactive O_2^- and H_2O_2 to a highly reactive $HO^\bullet$ (Fenton reaction) [50,51]. $HO^\bullet$ oxidizes membrane phospholipids, which compose cells and intracellular organelles, and iron forms radicals from produced peroxide lipids, thereby enhancing lipid peroxidation. Iron site-specifically combines with DNA and promotes DNA damage caused by ROS. Iron also increases ROS production by CYP2E1 [52]. A report that an enhanced peroxidation of intrahepatic lipids is attenuated by exsanguination in hemochromatosis also supports the involvement of iron in oxidative stress [53].

An excessively high iron content in the liver was observed in chronic hepatitis C [8,54]. Other investigators reported that iron removal therapy by exsanguination of chronic hepatitis C patients significantly improves serum ALT level without affecting viral load [55–57]. Another study showed that hepatic impairment is exacerbated following the administration of iron to chimpanzees with chronic hepatitis C [58]. Furthermore, oxidative stress is decreased by the iron removal therapy for chronic hepatitis C using intrahepatic 8-OHdG level as an index [57]. The above-mentioned reports show the close relationships of chronic hepatitis C with iron metabolism and oxidative stress.

8. Interactions with alcohol

Alcohol metabolism plays an important role in ROS production. Mainly alcohol dehydrogenase (ADH) in the cytosol and CYP2E1 (microsomal ethanol-oxidizing system) in microsomes are responsible for alcohol metabolism in the liver. When alcohol dehydrogenase oxidizes ethanol to acetaldehyde, the reduction from NAD^+ to NADH simultaneously occurs. NADH accumulation causes stress on the mitochondrial electron transfer system, leading to an increased production of ROS [59]. NADH also inhibits xanthine dehydrogenase activity, and xanthine is thereby oxidized by xanthine oxidase with the production of ROS [60]. CYP2E1 is induced by chronic alcohol intake and ROS are produced when CYP2E1 oxidizes ethanol to acetaldehyde [52,61].

There is no significant difference in hepatic peroxide level between core gene transgenic mice at 3–6-months old and control transgenic mice, but hepatic peroxide level significantly increases following the administration of a low dose of alcohol in the core gene transgenic mice [2]. ROS production increases upon glutathione reduction in HepG2 cells, with the co-expression of the core protein and CYP2D1, the latter of which is induced by alcohol [62]. These findings show that the core protein and alcohol in combination increase oxidative stress. Indeed, it was reported that alcohol intake plays a role in promoting the progression of chronic hepatitis C [63,64] and that increased levels of oxidative stress markers such as HNE and lipid hydroperoxide also support these findings [65]. From the viewpoint of oxidative stress also, HCV infection and alcohol intake are both considered to promote hepatic impairment.

9. Hepatocarcinogenesis and oxidative stress

It has been demonstrated that oxidative stress plays a key role in carcinogenesis [66,67]. Animal experiments using hepatocarcinogenesis models with the administration of a chemical substance (diethyl-nitrosamine, peroxisome proliferators) and with the administration of a choline-deficient

amino acid diet also indicates the involvement of oxidative stress [68–72]. In Long Evans Cinnamon (LEC) rats, an animal model that spontaneously develops heritable hepatitis and HCC caused by an abnormal copper accumulation, a congenitally decreased glutathione peroxidase expression level was reported, and the close relationship between oxidative stress and hepatocarcinogenesis was indicated [73].

The epidemiological relationship between HCV infection and HCC is evident [74,75], but the mechanism underlying this relationship has not been fully elucidated yet. Among postulated hypotheses on the mechanism of HCV-associated hepatocarcinogenesis, that of the involvement of the viral protein, in particular, the core protein of HCV is attractive: HCC develops in core gene transgenic mice, and carcinogenesis starts with well-differentiated carcinoma with an excessively high fat content, similar to hepatocarcinogenesis in human chronic hepatitis C, and poorly differentiated carcinoma with a low fat content develops in the form of "nodules in nodules" [76]. Because oxidative stress is increased in the core gene transgenic mice as mentioned above, it is assumed that oxidative stress plays an important role in hepatocarcinogenesis in chronic hepatitis C. Because the development of HCC is also observed in transgenic mice carrying the full-length HCV protein gene, the non-structural protein may have an additive effect to the effect of the structural proteins including the core protein, contributing to hepatocarcinogenesis [77]. NS5A, which was also reported to induce ROS production [4], may also contribute to hepatocarcinogenesis, although ROS induction by NS5A is not unequivocally confirmed yet.

Mitochondrial DNA has no potent protective proteins such as histone and is near the electron transport system, the major ROS production site. Hence, it is 10 to 15 times more susceptible to mutation caused by ROS than nuclear DNA [28,29]. In an investigation of mitochondrial DNA mutation in the human normal liver, both cancerous and non-cancerous liver tissues in patients with HCC showed very high incidences of DNA mutations [78]; thus, a relationship between oxidative stress persistence and hepatocarcinogenesis is suggested.

In the core protein expression system in the hepatic tissue and cultured cells of core gene transgenic mice, the activation of transcription factor AP-1 via mitogen-activated protein (MAP) kinase was observed [79–83]. The activation of the transcription factors AP-1, NF-κB, and signal transducer and activator of transcription (STAT) 3 by NS5A were also reported [4,84]. The activation of these transcription factors may facilitate cell proliferation, contributing to tumorigenic transformation.

It was also reported that ROS facilitate apoptosis via c-Jun N-terminal kinase (JNK)/p38 MAP kinase or by directly attacking the mitochondria. Apoptosis is a protective mechanism of the host against viral infection and carcinogenesis. Some reports stated that the core protein facilitates apoptosis [85–88], whereas other reports stated that the core protein inhibits apoptosis [89–92]; thus, no fixed view has yet been established. If it indeed inhibits apoptosis, it is assumed that this inhibition proceeds by maintaining oxidative stress and that the core protein has a beneficial effect against carcinogenesis and persistent viral infection.

In HCV infection, viral proteins such as the core protein and, possibly, NS5A protein induce oxidative stress, intracellular signaling, and transcription factors, which are not reflected in blood ALT level, contributing to the progression of carcinogenesis. Carcinogenesis, however, is slow as is observed in humans and core gene transgenic mice, the latter of which developed HCC in the latter half of their life. Recently, Okanoue et al. reported a long-term follow-up study of subjects with persistent HCV infection who had persistently normal ALT levels (PNAL) [93]. In their study, serum thioredoxin levels were not elevated in those with PNAL compared to those with chronic hepatitis. This may apparently seem contradictory to the results of our above-mentioned animal model studies. However, we should realize that anti-oxidant system is also instrumental in the liver. In these relatively younger people with PNAL than those with CH [93], active anti-oxidant system may erase the apparent elevation of ROS. Such a phenomenon was described in a mouse model by Moriya et al. [2], in which ROS was apparently normal in young core gene transgenic mice with the activation of catalase and reduction of GSH. Clinically, the presence of inflammation is thought to facilitate the process of hepatocarcinogenesis.

10. Conclusions

A very close pathological relationship between oxidative stress and HCV infection is observed, as shown by the above overview of relevant publications and discussion. The causes of oxidative stress in HCV infection are considered to include various factors such as mitochondrial damage, endoplasmic reticulum stress, iron accumulation, and lipid accumulation in the liver. Various study results demonstrated that even only viral proteins, mainly the HCV core protein, cause oxidative stress. When inflammation via immunoreactions to viral infection is added to oxidative stress, ROS production is expected to further increase, leading to a state in which the anti-oxidation system cannot cope with. In this sense, inflammation in chronic hepatitis C is considered to be qualitatively different from inflammation observed in other types of hepatitis such as autoimmune hepatitis or hepatitis B [94] (Fig. 5). As a treatment of chronic hepatitis C, the eradication of the virus is ideal. If it is not possible, however, the control of factors that exacerbate oxidative stress, such as inflammation via immune reaction and alcohol, and the relief of oxidative stress by the iron removal therapy and the administration of an anti-oxidation agent are considered to delay the progression of chronic hepatitis.

The development of such new anti-oxidation agents is being awaited. In further studies on the development of new therapies for hepatitis C and control methods for hepatocarcinogenesis in the future, the importance of those focusing on oxidative stress is expected to markedly increase.

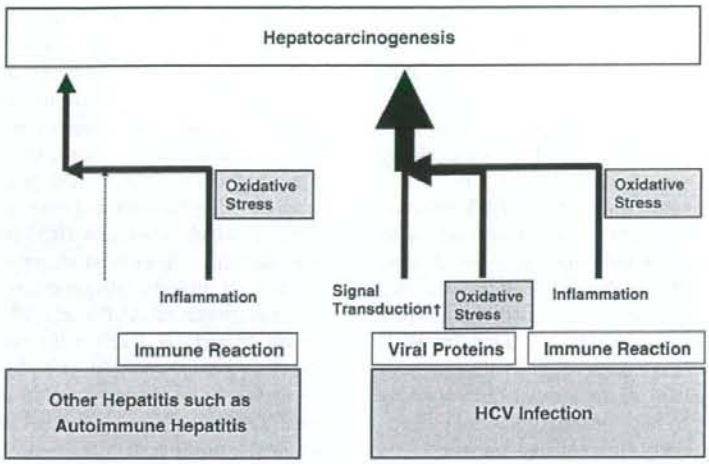

Fig. 5. Oxidative stress and hepatocarcinogenesis in various types of hepatitis (hypothesis). Oxidative stress is generated in all types of hepatitis via inflammation accompanied by continual cell death and regeneration. In HCV infection, HCV itself causes the production of oxidative stress in a synergy with inflammation. In this sense, the quality of "inflammation" in HCV infection may be different from that in other types of hepatitis. Additional impact of HCV proteins on the intracellular signal transduction would provoke the development of HCC. These may explain the conspicuous properties of HCC development.

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Journal of Gastroenterology

Editorial

Oxidative stress and apoptosis in hepatitis C: the core issue

Article on page 257

Hepatitis C virus core protein inhibits deoxycholic acid-mediated apoptosis despite generating mitochondrial reactive oxygen species

Hara Y, Hino K, Okuda M, et al.

Extra- as well as intracellular stimuli elicit a wide range of responses, such as cell proliferation, differentiation, survival and apoptosis, via the regulation of intracellular signaling. Recent studies have revealed that stress-responsive signal transduction pathways are stringently regulated by the intracellular redox state.[1] The redox state of the cell is determined by the delicate balance between the levels of oxidizing and reducing equivalents, including reactive oxygen species (ROS) and endogenous antioxidants. The production of ROS, a representative of oxidative stress, fluctuates in response to alterations in both external and internal environments and, in turn, triggers specific signaling cascades, such as mitogen-activated protein kinases, which determine cell survival or death. Thus, ROS are profoundly involved in cell death or apoptosis.

In the liver, ROS are also key cytotoxic and signaling mediators in the pathophysiology of liver diseases, including viral hepatitis, in which hepatocytes and resident and infiltrating phagocytes can generate ROS. While ROS are able to cause cell death through massive lipid peroxidation, they also act to modulate signal transduction pathways by affecting redox-sensitive enzymes, transcription factors, and organelles, including mitochondria and endoplasmic reticulum. ROS, thus, directly regulate apoptotic and necrotic cell death.[2] In addition, ROS have indirect effects on the pathophysiology of cell death by supporting protease activity via inactivation of antiproteases.

In hepatitis C virus (HCV) infection, both ROS and apoptosis are closely involved in the process of progressive liver diseases from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC).[3–7] ROS are assumed to play a major role in the pathogenesis of chronic hepatitis, which is characterized by continual cell death followed by regeneration. In this condition, the viral proteins of HCV, the core and nonstructural (NS) 5A proteins, have been shown to play a role in inducing ROS as well as in modulating apoptosis of hepatocytes.

The HCV genome comprises the genes of four structural proteins and six nonstructural proteins, and at least two of these viral proteins have been reported to cause oxidative stress in cells. The core protein, a structural protein, has been found to have various actions, including the induction of oxidative stress and the accumulation of lipids, in experimental studies using cultured cells and transgenic mice.[7,8] Experiments using mice transgenic for the core gene showed increased ROS production, increased intrahepatic catalase activity, a decreased intrahepatic glutathione (GSH) level, and a decreased GSH/GSH-GSSG (dimeric oxidized glutathione) ratio, indicating inhibition of antioxidation effects, although there was neither an increase in the serum alanine aminotransferase (ALT) level nor a histological finding of hepatitis.[7] Increased levels of intrahepatic peroxide lipids in the core gene transgenic mice with aging, compared with levels in control mice, also indicate an increase in oxidative stress. One possible mechanism underlying oxidative stress induction by the core protein is mitochondrial damage. Morphological abnormalities of the mitochondria have been observed in core gene transgenic mouse liver,[7] and increased ROS production caused by damage to the mitochondrial electron transport system has been noted in core protein-expressing cells.[8] Mitochondrial DNA, which has no protective proteins such as histone, is susceptible to damage by ROS. Mitochondrial DNA damage in the core gene transgenic mice appeared when they were as young as 3 months old. This mitochondrial damage disrupts the synthesis of proteins constituting the electron transport system complex and might also increase oxidative stress caused by damage to the electron transport system.

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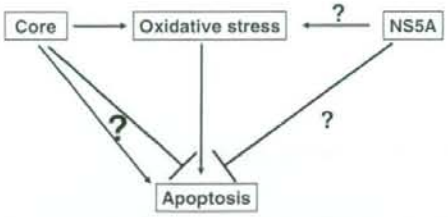

Fig. 1. Relationships among oxidative stress, apoptosis, and HCV proteins

A study using a cell culture system demonstrated that the NS5A protein also causes oxidative stress. NS5A induces the endoplasmic reticulum to release calcium by causing stress to the endoplasmic reticulum, and this release leads to increased ROS production in the mitochondria.[9] Although the effect of NS5A has not yet been confirmed by other study groups, HCV has the direct action of increasing intracellular ROS production via its proteins, separate from the induction of oxidative stress as a result of inflammation caused by viral infection (Fig. 1). A report that oxidative stress is also observed in HCV carriers with a normal ALT level[10] indicates that it is induced directly, without any mediating inflammatory reactions being necessary.

In contrast to the production of ROS by the core protein, which is now quite evident, the role of HCV core protein in apoptosis is rather controversial. Regarding the HCV-induced apoptotic mechanism, the HCV core protein may have a regulatory function in modulating apoptosis, either by enhancing or inhibiting it. In particular, the core protein exhibits both proapoptotic and antiapoptotic actions, depending on experimental conditions and the type of cells used,[11–14] whereas both the NS3 and the NS5A proteins have antiapoptotic effects (Fig. 1).[15] Modulation of apoptosis may involve binding of the core protein to the intracellular signal transducing portion of death receptors such as TNF-α, Fas, or lymphotoxin-αβ. Thus, HCV proteins may modulate hepatocyte apoptosis by indirect rather than by direct mechanisms. The real role of the core protein in the apoptotic process is, thus, not defined yet.

In the current issue of *Journal of Gastroenterology*, Hara et al.[16] tried to elucidate this core issue of HCV pathogenesis by separating the two properties of the HCV core protein with cultured cells, Huh-7 and HeLa. They confirmed that the core protein induced ROS, which was followed by activation of the scavenging system and insults to the cellular DNA, as shown previously.[6] In the study by Hara et al.,[16] the core protein inhibited the proapoptotic action of deoxycholic acid (DCA), which is known to cause both ROS production and apoptosis. Thus, the core protein seems to act to oppose the proapoptotic function of ROS, which ROS are also induced by the core protein itself. Such apparently opposing actions of the core protein, the production of ROS and the inhibition of apoptosis, might well explain the mode of hepatocarcinogenesis in HCV infection: hepatocytes with ROS-induced DNA damage may evade apoptosis by another effect of the core protein that inhibits apoptosis. Such a mechanism, similar to one previously postulated by other researchers,[3] in which both ROS production and mitogen-activated protein kinase activation are ascribed to the core protein, may clarify how cells with DNA damage can survive and develop into buds of HCC. Regrettably, the current study was done using DCA as an agent to induce both apoptosis and ROS, making it difficult to interpret the authentic role of the core protein in the execution of such biological functions. As noted above, the effect of the core protein in apoptosis varies depending on the system used. Therefore, further studies using different systems may be necessary to bring a conclusion to the core issue of HCV-induced pathogenesis associated with the multipotential HCV core protein.

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RAPID COMMUNICATION

Risk factors for retinopathy associated with interferon α-2b and ribavirin combination therapy in patients with chronic hepatitis C

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Abstract

AIM: To elucidate the frequency and risk factors for retinopathy in patients with chronic hepatitis C who are treated by interferon-ribavirin combination therapy.

METHODS: We prospectively analyzed 73 patients with histologically confirmed chronic hepatitis C, who underwent combination therapy for 24 wk. Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy.

RESULTS: Fourteen patients (19%) developed retinopathy, which was initially diagnosed by the appearance of a cotton wool spot in 12 patients. Retinal hemorrhage was observed in 5 patients. No patient complained of visual disturbance. Retinopathy disappeared in 9 patients (64%) despite the continuation of combination therapy. However, retinopathy persisted in 5 patients with retinal hemorrhage. A comparison of the clinical background between the groups with and without retinopathy showed no significant differences in age, gender, viral genotype, RNA level, white blood cell count, platelet count, prothrombin time, complications by diabetes mellitus or hypertension, or pretreatment arteriosclerotic changes in the optic fundi. However, multiple logistic regression analysis revealed that complication by hypertension was observed with a high frequency in the group with retinopathy (P = 0.004, OR = 245.918, 95% CI = 5.6-10786.2).

CONCLUSION: Retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

Key words: Retinopathy; Ribavirin; Chronic hepatitis C; Interferon

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INTRODUCTION

Chronic hepatitis C, which affects more than 170 million people in the world[1], may eventually lead to cirrhosis and/or hepatocellular carcinoma. The main treatment for this intractable disease is interferon administration. Published guidelines recommend interferon-ribavirin combination therapy as a first-line treatment[2]. Interferon is also used in the treatment of other viral and neoplastic diseases.

Various adverse effects have been reported due to use of interferon[3]. An influenza-like syndrome, characterized by fever, chills, myalgias, arthralgias, and headache, is the most common adverse effect. Toxicities of the central nervous, hematopoietic, gastrointestinal, urinary, cardiovascular, musculoskeletal and endocrine systems have also been described. However, ocular toxicity was not reported before the use of interferon for chronic hepatitis[3].

After the introduction of interferon for the treatment of hepatitis, retinal complications have been reported. Hayakawa *et al* showed that 17 of 43 patients developed retinopathy during interferon monotherapy. They also showed that the prevalence of retinopathy was higher in patients with diabetes[4]. Subsequently, several papers have shown that a substantial proportion of patients undergoing interferon monotherapy develop retinopathy[5-7]. However, the prevalence of retinopathy is variable, which is

presumably attributed to the difference in the treatment regimen and/or background of patients.

As mentioned above, interferon-ribavirin combination therapy has become the standard treatment for chronic hepatitis C. Results from recent studies have suggested that the prevalence of retinopathy associated with combination therapy may be higher than that associated with interferon monotherapy, which should be further investigated[8-10].

In spite of the high prevalence, risk factors for interferon-associated retinopathy are still unclear. Diabetes mellitus and the patients' age were reported to be possible risk factors for retinopathy associated with interferon monotherapy[4]. In interferon-ribavirin combination therapy, diabetes, hypertension[8], and response to treatment[10] were considered possible risk factors. However, the results are not conclusive because of the small number of patients examined.

The aim of the present study is to elucidate the prevalence and risk factors for retinopathy associated with interferon-ribavirin combination therapy.

MATERIALS AND METHODS

Patients

Seventy-three consecutive patients with histologically confirmed chronic hepatit is C (47 males and 26 females; median age, 53.4 years; ranges 26-73 years) were enrolled in this study from 2002 to 2004. The clinical backgrounds of the enrolled patients are shown in Table 1. All patients were treated with recombinant interferon α-2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ, USA) combination therapy. All the patients were treated daily with interferon α-2b at 6 MU for 2 wk followed by three times a wk treatment with interferon α-2b at 6 MU for 22 wk in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for patients who weighed 60 kg or less and 800 mg for the remaining patients who weighed more than 60 kg for 24 wk.

All patients were assessed to determine the safety, tolerance, and efficacy of the treatment at the end of wk 1, 2, 4, and every 4 wk during the treatment. After the treatment was completed, patients were followed up on wk 4, 8, 12, and 24. The primary end point was indicated by a sustained loss of detectable HCV-RNA at 24 wk after the treatment.

Methods

Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy. Ophthalmological examinations were carried out before the start of treatment and 2, 4, 12 and 24 wk after the start of treatment until the completion of treatment or until the retinopathy disappeared. Fundus photographs were taken for documentation and comparison when retinal abnormalities were detected.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of our institutions.

Table 1 Profiles and initial laboratory data of patients with and without retinopathy during IFN-ribavirin combination therapy[1]

	Total	Retinopathy (+)	Retinopathy (-)
Patients			
Number	73	14	59
Age (yr)	53.4 ± 10.9	56.3 ± 10.5	52.8±38.6
Gender (M/F)	47/26	10/4	37/22
Hypertension (Yes/No)*	15/58	5/8	10/49
Diabetes mellitus (Yes/No)	2/71	1/13	1/58
Peripheral blood count			
Platelet count ($\times 10^4$/mm^3)	15.3 ± 6.0	12.5 ± 10.5	15.9 ± 38.6
White blood cell ($\times 10^3$/mm^3)	46.9 ± 12.6	46.5 ± 13.0	48.6 ± 10.9
Hemoglobin (×g/dL)	14.0 ± 1.3	14.0 ± 1.0	14.0 ± 1.4
Prothrombin time (%)	90.2 ± 13.3	87.1 ± 13.3	90.8 ± 13.3
ALT (IU/L)	109.4 ± 78.2	104.1 ± 41.0	110.4 ± 83.6
Viral factors			
Genotype (type 1/type 2)[2]	45/26	33/24	12/2
Viral load (kcopies/mL)	592.3 ± 271.2	505.6 ± 309.1	607.5 ± 271.2
Pretreatment/ Arteriosclerotic changes in optic fundi (Yes/No)	12/61	7/7	5/54
Response to therapy (SVR/non-SVR)	38/35	5/9	33/26

[1] Data are expressed as mean ± SD.
[2] Genotype could not be determined in 2 patients.
*$P = 0.004$

RESULTS

Before the start of the combinaiton therapy, one patient had scars from laser coagulation of a previous interferon-associated retinopathy and another patient had retinal central vein occlusion. Arteriosclerotic changes of the optic fundi were observed in 12 patients.

After the start of interferon-ribavirin combination therapy, 14 out of 73 patients (19%) developed retinopathy. The clinical profiles and laboratory data of the patients with and without retinopathy are shown in Table 1.

We compared the characteristics of patients who developed retinopathy and those who did not. The two groups showed no statistical differences in age, gender, subtype of virus, RNA level, white blood cell count, platelet count, prothrombin time before treatment or prevalence of pretreatment fundic arteriosclerotic changes. The patients with retinopathy were more frequently complicated by hypertension ($P = 0.004$) (Table 1).

Logistic regression analysis of factors affecting retinopathy was also carried out. Hypertension was found to be a factor for predicting retinopathy (Table 2).

Table 3 shows the optic fundi findings of the 14 patients with retinopathy. Retinopathy was initially diagnosed by the appearance of a cotton wool spot in 12 patients. In three of the 12 patients, retinal hemorrhage was also observed simultaneously or sequentially. Two of the 14 patients who developed retinopathy were diagnosed by retinal hemorrhage without a cotton wool spot. No patient complained of the visual disturbance.

Table 2 Logistic regression analysis of factors associated with retinopathy

Factor	P	Odds ratio	95% confidence interval
Sex	0.68	1.699	0.1-21.0
Age	0.203	1.099	1.0-1.3
Genotype	0.776	1.621	0.1-45.5
Levels of HCV RNA	0.114	1.006	0.99-1.0
Hypertension	0.004	246.32	5.5-10977.8
Diabetes mellitus	0.211	0.122	0.1-3.3
Abnormal findings in pretreatment optic fundi	0.904	1.192	0.1-20.3
Platelet	0.059	1.391	1.0-1.9
Prothrombin time	0.747	0.982	0.9-1.1
ALT	0.992	1	0.98-1.0
WBC	0.964	1.027	0.4-2.9
Response to therapy (SVR or non-SVR)	0.123	0.016	0.0-3.1

Table 3 Optic fundi findings of patients with retinopathy

No	Age	Sex	Underlying disease		Optic fundi before treatment		Optic fundi after treatment	
			Hypertension	Diabetes mellitus	H	S	Cotton wool spot	Retinal hemorrhage
1	38	M	+	+	0	0	4 wk-	4 wk-
2	52	M	+	-	1	0	4-12 wk	-
3	40	M	-	-	0	0	6-36 wk	-
4	62	F	-	-	0	0	4-36 wk	-
5	61	M	+	-	0	0	12 wk-	-
6	58	M	-	-	1	1	12 wk-	-
7	73	M	-	-	2	2	4-28 wk	-
8	65	F	+	-	0	0	24-36 wk	-
9	59	F	+	-	2	2	2 wk-	4-24 wk
10	40	M	-	-	0	0	4-20 wk	-
11	62	F	-	-	1	2	2 wk-	4 wk-
12	65	M	-	-	1	1	2-24 wk	-
13	40	M	-	-	0	0	-	8-16 wk
14	40	M	-	-	0	0	-	2-4 wk

Retinopathy disappeared in 9 of the 14 patients despite the continuation of combination therapy. However, it continued in three patients with retinal hemorrhage and two without retinal hemorrhage.

Ocular manifestations other than retinopathy (e.g., ocular pain, a mild watery eye and conjunctivitis) were not observed in any patients.

DISCUSSION

Interferon associated retinopathy was first recognized in 1990 when Ikebe and associates reported a 39-year-old patient who developed retinal hemorrhages and cotton wool spots following intravenous administration of interferon[11].

The exact mechanism of interferon-induced-retinopathy is not known but is presumably related to the disturbance in retinal microcirculation[12]. Therefore, preexisting arteriosclerosis that affects microcirculation may promote interferon-induced retinopathy.

Our study shows that hypertension is a more frequent complication in patients with interferon-induced-retinopathy. Chronic hypertension is associated with the thickening of the walls of the arteries and small arterioles[13]. Therefore, systemic hypertension predisposes patients to interferon-induced-retinopathy. The fact that hypertensive retinopathy induces the formation of flame-shaped hemorrhages and white cotton wool spots, which are also seen in interferon-induced-retinopathy, implies that systemic hypertension and interferon-induced-retinopathy may be related each other.

Statistical analysis did not indicate pretreatment optic fundic changes or diabetes as predictive factors of retinopathy. This may be attributed to the following reasons: (1) pretreatment changes in the optic fundi as a predictive factor are included in hypertension; and (2) the number of patients with diabetes is too small. Regardless of these reasons, systemic hypertension is an important risk factor for interferon-related retinopathy.

The frequencies of interferon-induced retinopathy associated with interferon monotherapy and interferon-ribavirin combination therapy are reported to be 24%-58%[4-7,14,15] and 16%-64%[8-10,16], respectively. The frequency in the present study (20%) was lower than that in previous reports. Furthermore, the ocular side effects of ribavirin, which include a mild watery eye and conjunctivitis, were not seen in this study. Therefore, the frequency of induced retinopathy associated with combination therapy may be considered as high as that associated with interferon monotherapy.

Retinopathy developed by 12 wk in most (13/14, 93%) of the patients after the start of combination therapy and disappeared in majority (10/14, 71%) of the patients during the 4-8 wk period, in which the patients were receiving the treatment. This suggests that treatment can be continued despite the development of retinopathy in many patients. However, two patients who developed cotton wool spots early in the therapy (2 wk) therafter suffered from retinal hemorrhage in a prolonged manner. Therefore, patients who develop cotton wool spots early in the therapy should be carefully monitored. However, as reported in previous studies[4,8,17], most of the patients with retinopathy in this study were asymptomatic. Therefore, combination therapy may be continued in most patients.

The fact that retinopathy occurred more frequently in patients with hypertension, suggests that these patients should be carefully monitored. With periodic examination of the optic fundi, major bleeding that causes visual symptoms may be prevented or detected at an early stage. Therefore, patients who undergo interferon-ribavirin combination therapy, particularly those with hypertension, should undergo periodic examination of the optic fundi.

To conclude, retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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Cooperative contribution of gag substitutions to nelfinavir-dependent enhancement of precursor cleavage and replication of human immunodeficiency virus type-1

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Abstract

We previously described a clinical human immunodeficiency virus type-1 (HIV-1) isolate, CL-4, which showed nelfinavir (NFV)-dependent enhancement of replication (Matsuoka-Aizawa, S., Sato, H., Hachiya, A., Tsuchiya, K., Takebe, Y., Gatanaga, H., Kimura, S., Oka, S, 2003. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J. Virol. 77, 318–327.). To identify the responsible region(s) of HIV-1 proteins for such replication enhancement, we constructed a panel of recombinant HIV-1 clones harboring portions of the Gag and protease of CL-4 and analyzed their replication capabilities and Gag processing patterns. Our data suggested that the substitutions in the matrix and N-terminal half of capsid of CL-4 were indispensable for the NFV-dependent enhancement of replication and that NFV facilitated the cleavage between the matrix and capsid of the Gag precursor harboring these substitutions. The substitutions in C-terminal half of capsid rather decreased the cleavability of Gag precursor and NFV counteracted such negative impact. Efficient replication enhancement with NFV can be observed only in the presence of the substitutions in entire Gag and protease of CL-4.

Keywords: Human immunodeficiency virus type 1; Nelfinavir-resistant; Gag mutation

1. Introduction

Under the selective pressure of antiretroviral agents, the human immunodeficiency virus type-1 (HIV-1) evolves and acquires drug-resistance-associated mutations. The major protease inhibitor (PI)-resistance-associated mutations are located in the active sites of HIV-1 protease and impair its enzymatic functions (Bleiber et al., 2001; Croteau et al., 1997; Martinez-Picado et al., 1999). In order to compensate such impaired enzymatic function, PI-resistant HIV-1 further acquires mutations not only in protease but also in one of its substrate, Gag, resulting in full recovery of replication ability (Doyon et al., 1996; Gatanaga et al., 2002; Tamiya et al., 2004; Zhang et al., 1997). We previously described a unique clinical HIV-1 isolate, CL-4, which replicated more efficiently in the presence of sub-inhibitory concentrations of nelfinavir (NFV) (0.001–0.1 μM) (Matsuoka-Aizawa et al., 2003). CL-4 had a total of 56 amino acid substitutions in *gag-pro* genes compared with NL4-3; 22 substitutions had emerged in the matrix, SP1, and protease during administration of NFV-containing therapy, and 34 other substitutions had already existed before the introduction of the therapy (Matsuoka-Aizawa et al., 2003). In that study, we constructed three HIV-1 clones including, p17PRmt, PRmt, and p24PRmt, and found that only p17PRmt, which possessed the entire Gag and protease segment of CL-4, showed NFV-dependent enhancement of replication. Therefore, we concluded that the substitutions in matrix are indispensable for replication enhancement (Matsuoka-Aizawa et al., 2003). However, it is still unknown whether the substitutions in matrix alone are sufficient or whether other Gag substitutions are necessary for the replication enhancement with NFV. In this study we constructed four more recombinant HIV-1 clones and characterized their replica-

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tion kinetics and Gag processing in the absence and presence of NFV.

2. Materials and methods

2.1. Cells and antiretroviral agents

HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transformed T cell lines, MT-2, PM-1, and H9 cells were maintained in RPMI-1640 with 10% FCS. NFV was kindly provided by the Japan Tobacco Co. (Tokyo, Japan).

2.2. Plasmid construction and preparation of gag-pro recombinant HIV-1 clones

Clinical HIV-1 isolates CL-1, CL-2, CL-3, and CL-4 were sequentially obtained from the same patient before and during NFV-containing treatment (Matsuoka-Aizawa et al., 2003). Direct sequences of these four clinical isolates and sub-cloning

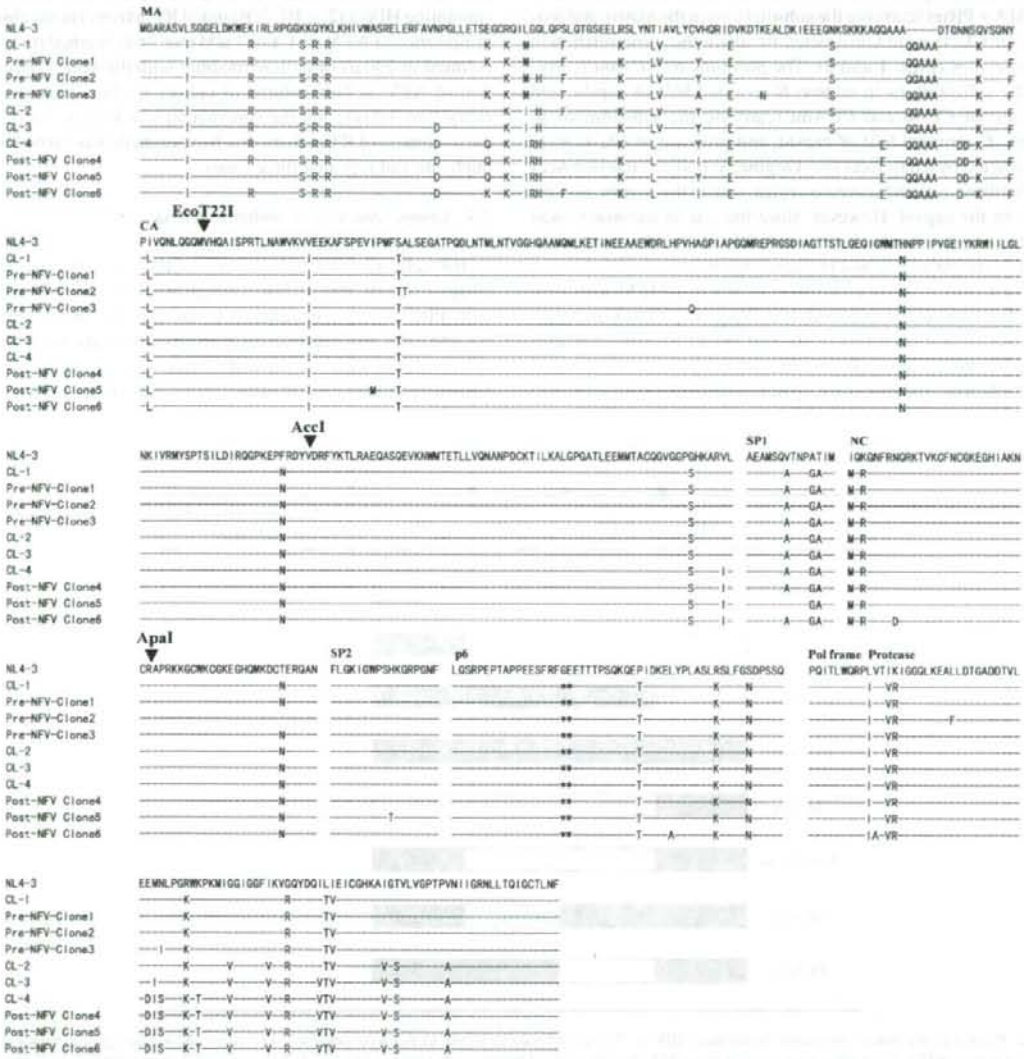

Fig. 1. Direct and sub-clonal sequences of clinical HIV-1 isolates. Direct and sub-clonal amino acid sequences of whole Gag and protease of HIV-1 isolates are shown. Pre-NFV Clone 1–3 and Post-NFV Clone 4–6 were derived from CL-1 and CL-4, respectively. The amino acid sequence of HIV-1_{NL4-3} is shown at the top as reference. The identical amino acids with those of HIV-1_{NL4-3} are indicated with dashes and the star shows deletion compared with HIV-1_{NL4-3} sequence. The restriction sites used in the construction of recombinant HIV-1 plasmids are also shown. MA, matrix; CA, Capsid; NC, nucleocapsid; and PR, protease.

sequences of CL-1 and CL-4 indicated that 11 and 10 amino acid substitutions accumulated in Gag and protease during PI-containing treatment, respectively (Fig. 1). Post-NFV Clone 4 (Fig. 1) was used in the construction of CL-4-derived recombinant HIV-1 plasmid. The pNL4-3-based plasmids of PRmt (HIV-1 carrying only the substitutions in protease of CL-4), p24PRmt (carrying the substitutions in capsid and protease of CL-4), and p17PRmt (carrying the substitutions in whole Gag and protease of CL-4) were constructed as previously described (Matsuoka-Aizawa et al., 2003) (Fig. 2), and the plasmids of MAmt (carrying only the substitutions in the matrix of CL-4) and MA + PRmt (carrying the substitutions in the matrix and protease of CL-4) were constructed by using the same restriction enzyme sites (Figs. 1 and 2). The plasmids of NCAmt (carrying the substitutions in matrix, N-terminal half of capsid, and protease of CL-4) and CCAmt (carrying the substitutions in matrix, C-terminal half of capsid, and protease of CL-4) were constructed by using AccI site. Originally, pNL4-3 has two AccI site between *gag* and protease region, one in the matrix, and the other in the capsid. However, since the one in the matrix was extinct due to natural substitution in CL-4, the other in the capsid was unique in *gag* and protease region.

HeLa cells (5×10^5 cells) were grown in DMEM with 10% FCS for 24 h and transfected with 3 μg of pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNAs by using FuGINE 6 transfection reagent (Roche Diagnosis, Basel, Switzerland). The cells were incubated for 24 h, washed once with PBS, and cultured in 5 ml of culture medium. The culture supernatant containing virus was collected at 48 h after transfection, filtered, analyzed for RT activity (10432–17162 cpm/μM), and kept at −80 °C until use. The virus titer used for infection and Western blot analysis was adjusted with RT activity.

2.3. HIV-1 replication kinetics

The methods used to infect cells were described previously (Matsuoka-Aizawa et al., 2003). Briefly, MT-2, PM-1, and H9 cells (2×10^4) were infected with 200 μl of cell-free supernatant containing HIV-1 (2×10^5 ^{32}P cpm of RT activity) in the absence or presence of NFV (0.1 and 1 μM) for 16 h, washed once, and cultured in 200 μl of culture medium with the same concentration of NFV. A half volume of culture medium was changed every 2 or 3 days, and the supernatant was kept at −80 °C for measurement of RT activity. Each experiment was carried out in duplicate and repeated three times.

2.4. Competitive HIV-1 replication assay

H9 cells (2×10^5 cells) were incubated with two HIV-1 clones (each of 100 $TCID_{50}$) simultaneously for 16 h, washed with PBS twice, and cultured in the absence or presence of 0.1 μM NFV for 7 days. These infection periods were defined as a single passage. At the end of each passage, H9 cells were harvested and the culture supernatants were used to infect fresh

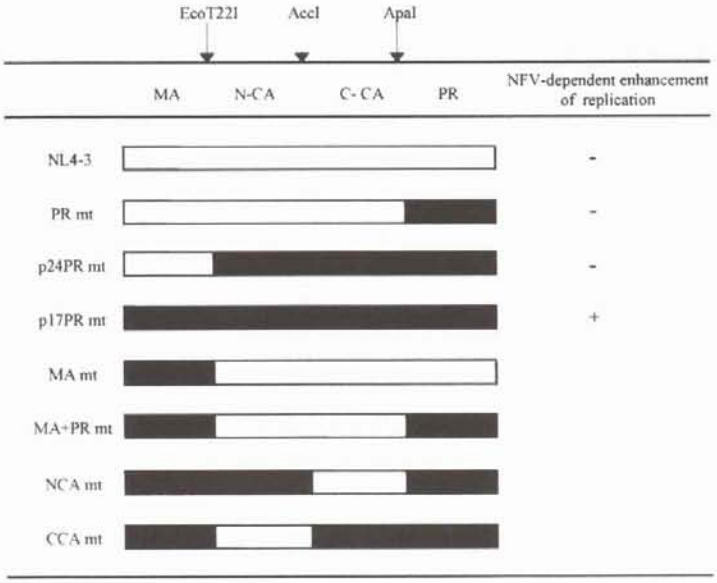

Fig. 2. Previously and newly constructed recombinant HIV-1s. The recombinant molecular clones were constructed based on pNL4-3 as a genetic backbone. The Gag-PR region of HIV-1 was segmented into four areas, MA (BssHII-EcoT22I fragment), N-terminal half of CA (NCA) (EcoT22I-AccI fragment), C-terminal half of CA (CCA) (AccI-ApaI fragment), and PR (ApaI-BalI fragment). Originally, pNL4-3 has two AccI sites between the gag and PR region, in MA and CA. However, because the one in MA was extinct in CL-4 due to natural substitution, the other AccI site in CA was unique for gag-PR gene of CL-4. Open boxes indicate the NL4-3-originated fragments, and closed boxes indicate fragments that were derived from CL-4 variants. The NFV-dependent replication enhancement of previously analyzed clones was also shown and indicated as (+). MA, matrix; CA, capsid; and PR, protease.

uninfected H9 cells. The cells harvested at each passage were subjected to PCR for amplification of HIV-1 *gag* region and direct DNA sequencing was performed. The viral populational changes were determined by relative peak height on sequence electrophoregram (Kosalaraksa et al., 1999).

2.5. HIV-1 susceptibility to NFV

MT-2 cells were infected with 500 $TCID_{50}$ of each virus in the absence and the presence of 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.316, 1, and 3.16 μM of NFV, and cultured in triplicate for 7 days. At the end of culture, the amounts of p24 in the supernatants were measured and 50% inhibitory concentrations (IC_{50}) of NFV were determined by referring to the dose–response curve.

2.6. Western blot analysis of HIV-1 virions

HeLa cells were transfected with pNL4-3 and *gag*-protease recombinant HIV-1 plasmid DNA in the absence and presence of 0.1 μM NFV. The culture supernatant was harvested at 48 h after transfection, centrifuged at 37,000 × *g* for 90 min to pellet virus particles. The virion pellet (6×10^5 cpm of RT activity) was applied to an SDS gradient gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with anti-HIV-1 p24 antisera (Advanced Biotechnology, Columbia, USA) and HIV-1-infected patients' serum, respectively, and hybridized with anti-protein A antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The immune complex was visualized with an ECL Plus system (Amersham Pharmacia Biotech) according to the manufactures' description.

The percent signal density of Gag products was analyzed on a Windows computer by using the ImageJ Program (developed at the U.S. National Institutes of Health (http://www.rsb.info.nih.gov/ij/)) and the percent density of p24 was determined by the following formula: percent density of p24 = 100 × (the density of p24 signal)/(the cumulated density of all *Gag* signals) (Tamiya et al., 2004).

3. Results

3.1. Whole capsid substitutions necessary for NFV-enhanced replication

MAmt, carrying only the substitutions in the matrix (Fig. 2), grew well in the absence of NFV (Fig. 3). In the presence of NFV, however, it did not grow at all, indicating that matrix substitutions were not sufficient to confer NFV resistance. MA + PRmt, carrying substitutions in the matrix and protease (Fig. 2), replicated as efficiently as PRmt (carrying only the substitutions in protease), both in the absence and presence of 0.1 μM NFV, though its replication was not enhanced with NFV, indicating that the substitutions in matrix and protease were not sufficient for NFV-dependent enhancement of replication. As reported in our previous study (Matsuoka-Aizawa et al., 2003), p17PRmt replicated more efficiently in the presence of 0.1 μM NFV than in the absence of NFV. Therefore, some of the substitutions in the capsid should be responsible for such unique phenotype of CL-4 strain. The HIV-1 capsid contains two domains, a C-terminal oligomerization domain and N-terminal core domain, which function differently in viral assembly (Turner and Summers, 1999). Therefore, we divided the EcoT22I—ApaI segment of CL-4 into two segments at ACC I site, named them the N-terminal half of the capsid (NCA) and the C-terminal half of the capsid (CCA), and constructed two recombinant HIV-1 clones, NCAmt and CCAmt, which possessed all the substitutions in the matrix and protease of CL-4, and the substitutions in NCA and CCA, respectively (Fig. 2). NCAmt and CCAmt grew efficiently both in the absence and presence of 0.1 μM NFV, and only NCAmt showed weak replication enhancement with 0.1 μM NFV in PM-1 and MT-2 cells though it was not so efficient as that of p17PRmt, suggesting that the substitutions in CCA, contributed to the efficient replication enhancement of p17PRmt (Fig. 3). CCAmt did not show the p17PRmt's phenotype, indicating that the substitutions in NCA were indispensable for replication enhancement. As we reported previously (Matsuoka-Aizawa et al., 2003), p24PRmt lacking the substitutions in matrix did not show replication enhancement by NFV. Taken together, the substitutions in the whole matrix, capsid, SP1, and the N-terminal end of nucleocapsid of CL-4 were indispensable for efficient replication enhancement of p17PRmt.

To define further the role of substitutions in the matrix, NCA, and CCA, viral replication efficiency was compared among the HIV-1 clones described above in the absence and presence of NFV using competitive HIV-1 replication assay (Kosalaraksa et al., 1999). MA + PRmt outgrew PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4a), and MAmt was outgrown by NL4-3 in the absence of NFV (Fig. 4b), suggesting that the substitutions in the matrix of CL-4 reduced the replication of HIV-1 harboring wild-type protease, but compensated the replication of HIV-1 harboring NFV-resistant protease of CL-4. NCAmt outgrew MA + PRmt both in the absence and presence of 0.1 μM NFV (Fig. 4c), suggesting that the substitutions in NCA were compensatory for the replication of HIV-1 harboring protease and matrix of CL-4. However, CCAmt was outgrown by MA + PRmt in the absence of NFV, but its replication in the presence of 0.1 μM NFV was comparable with that of MA + PRmt under similar condition (Fig. 4d), suggesting that the substitutions in CCA reduced the replication capability of MA + PRmt, while NFV compensated the mutation effect. Sub-cloning analyses of proviral sequences at both of the passages 3 and 4 in competitive HIV-1 replication assay in the presence of 0.1 μM NFV showed that five of 10 clones were derived from CCAmt and the other five clones were derived from MA + PRmt, which confirmed that CCAmt and MA + PRmt had comparable replication ability in the presence of 0.1 μM NFV (Fig. 4d). MA + PRmt readily outgrew p17PRmt in the absence of NFV, but was outgrown by p17PR in the presence of 0.1 μM NFV (Fig. 4e), suggesting that the substitutions in NCA and CCA reduced the replication capability of MA + PRmt, while NFV counteracted the mutation effect and rather enhanced replication ability at sub-inhibitory concentration (Fig. 3, p17PRmt).