Published in final edited form as:

Free Radic Biol Med. 2014 July ; 0: 267–284. doi:10.1016/j.freeradbiomed.2014.04.020.

Oxidative stress and hepatic Nox proteins in chronic hepatitis C and hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common liver cancer and a leading cause of cancer-related mortality in the world. Hepatitis C virus (HCV) is a major etiologic agent of HCC. A majority of HCV infections lead to chronic infection that can progress to cirrhosis and eventually, HCC and liver failure. A common pathogenic feature present in HCV infection, and other conditions leading to HCC, is oxidative stress. HCV directly increases superoxide and H₂O₂ formation in hepatocytes by elevating Nox protein expression and sensitizing mitochondria to reactive oxygen species generation while decreasing glutathione. Nitric oxide synthesis and hepatic iron are also elevated. Furthermore, activation of phagocytic NADPH oxidase 2 (Nox2) of host immune cells is likely to exacerbate oxidative stress in HCV-infected patients. Key mechanisms of HCC include: genome instability, epigenetic regulation, inflammation with chronic tissue injury and sustained cell proliferation, and modulation of cell growth and death. Oxidative stress, or Nox proteins, plays various roles in these mechanisms. Nox proteins also function in hepatic fibrosis, which commonly precedes HCC, and Nox4 elevation by HCV was mediated by transforming growth factor beta. This review summarizes mechanisms of oncogenesis by HCV, highlighting the role of oxidative stress and hepatic Nox enzymes in HCC.

Keywords

cancer; HCV; inflammation; NADPH oxidase; reactive oxygen species

I. Introduction

Oxygen is essential for the life of aerobic organisms. During aerobic respiration, O₂ is reduced to H₂O through a series of electron transfer reactions occurring in mitochondria that drive ATP synthesis. In addition, reactive oxygen species (ROS) are formed. While ROS are often associated with toxicity, not all biological ROS generation are harmful, and ROS participate in specific, reversible biochemical reactions in redox signaling. For example,

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reversible thiol oxidation of protein tyrosine phosphatases and peroxiredoxins by H_2O_2 that produces cysteine sulfenic acid intermediates is well-characterized (1). Even an over-production of reactive species is not necessarily harmful and could confer some benefit to the organism producing them. A clear example would be the burst of superoxide anion ($O_2^{\cdot-}$) produced by NADPH oxidase 2 (Nox2) in phagocytes during inflammation, which is critical for the destruction of microbes within phagosomes; absence of a functional Nox2 protein complex leads to chronic and frequent infections, as well as dysregulated inflammation in patients with chronic granulomatous disease (2). Nevertheless, aberrant levels of ROS that overwhelm the body's antioxidant defenses can result in pathogenic changes and tissue injury, and a shift in redox status is associated with diverse disease conditions.

Hepatitis C virus (HCV) is a major etiologic agent of severe liver diseases, including cancer. HCV perturbs the host redox status by increasing production of ROS and reactive nitrogen species (RNS), and oxidative stress has emerged as a common pathogenic feature in numerous *in vitro* and *in vivo* studies on HCV. Several sources of ROS contribute to hepatic oxidative stress during HCV infection, including hepatocyte and non-hepatocyte sources, and oxidative stress is likely to contribute to HCV-associated liver cancer through multiple mechanisms. This review summarizes the mechanisms of hepatocarcinogenesis by HCV, highlighting the role of oxidative stress and hepatic Nox enzymes in hepatocellular carcinoma (HCC).

A. Hepatocellular carcinoma

HCC is a malignant epithelial tumor of the parenchymal liver cell that accounts for 70 – 85 % of primary liver cancers (3). A majority of HCC cases occur in developing countries, with the highest prevalence in sub-Saharan Africa and Eastern Asia. HCC rates are low in developed countries, with the exception of Japan. HCC is the second leading cause of cancer-related mortality in the world (4). Disease prognosis is poor; five year survival rates of ~11 % have been reported, even in developed countries (5–7). Unlike many cancers that have declined over the years, HCC is still on the rise (8). In the U.S., the incidence rates of HCC tripled in both men and women from 1995 – 1997 to 2005 – 2007; death rates for liver cancer also increased (9). Increases in HCC incidence rates were most prominent among Hispanic, black, and white middle-aged men between 2000 – 2005 (9, 10). Higher overall HCC incidence and mortality rates were also found among Asians/Pacific Islanders compared to other groups tested, indicating significant health disparities among populations (9). A ten-fold increase in the prevalence of HCC was reported among U.S. military veterans with HCV infection from 1996 to 2006 (11). In 2009, 22,620 new cases of liver and intrahepatic bile duct cancers and 18,160 deaths were estimated in the U.S. (8). Worldwide, an estimated 748,300 new liver cancer cases and 695,900 liver cancer-related deaths occurred in 2008 (12).

Risk factors for HCC include viral hepatitis, alcohol abuse, aflatoxin exposure, obesity, as well as nonalcoholic fatty liver disease (4). Among these, viral hepatitis is the most important risk factor for HCC; together, hepatitis B virus (HBV) and HCV are responsible for 78 % of HCC worldwide (13). While the highest rates of HCC are found in areas where

HBV is endemic, the increasing incidence of HCC, particularly in developed countries, has been largely attributed to the prevalence of HCV infection. For example, HCV infection is found in approximately 80% of HCC cases in Japan and about half of HCC cases in the U.S. (14, 15). Unlike HBV, for which there is a vaccine, there is no vaccine that can prevent the spread of HCV infection, and this is likely contributing to the rising incidence of HCV-associated HCC. The odds ratio for developing HCC is 11.5 among HCV-infected individuals (16).

B. HCV induces HCC

HCV is an enveloped, positive sense RNA virus of the *Flaviviridae* family that was discovered in 1989 (17). HCV is transmitted through blood, and common risk factors for HCV infection include having received blood, blood products, or organs prior to 1992, injection drug use, birth to an infected mother, sex with an infected person, and occupational exposure. A majority of HCV infected individuals do not clear the virus and become chronically infected. It is estimated that approximately 170 million individuals are chronically infected with HCV worldwide, including 4 million in the U.S. (18, 19). Baby boomers, or people who were born between 1945 to 1965, are more likely to have hepatitis C than others; a National Health and Nutrition Examination Survey estimated that among the non-institutionalized, civilian U.S. population who had chronic HCV infection, approximately 66 % were baby boomers who would now have been living with chronic hepatitis C for several decades (20). It has also been estimated that approximately 50 % of individuals with chronic HCV infections are undiagnosed in the U.S. (20).

Chronic HCV infection occurs in about 80 % of HCV-infected individuals, which increases the risk of developing severe liver diseases. About 20 – 30 % of chronic hepatitis C patients develop cirrhosis in 20 – 30 years, and 4 – 7 % of these individuals progress to HCC and end stage liver disease each year (21). Extrahepatic manifestations, such as lymphoproliferative disorders, have also been associated with chronic HCV infection (22). HCV is the leading cause for liver transplantation in the U.S., and HCV-related deaths rose by 123 % between 1994 and 2005 in the U.S. (23). By 2020, the proportion of infected and untreated patients with cirrhosis is estimated to increase from 16 to 32 %, hepatic decompensation by 106 %, HCC by 81 %, and liver-related deaths by 180 % (24).

The previous standard of care for HCV infection, consisting of pegylated interferon (IFN) alpha and ribavirin, achieved sustained virological response (SVR) in 50 – 60 % of individuals undergoing treatment and produced severe side effects (25). SVR was as low as 30 % in patients with genotype 1, the most prevalent HCV genotype, and a high viral load. Recently developed direct-acting antivirals that target specific HCV factors, such as NS3/4A protease and NS5A protein, have shown strong promise, and these drugs are rapidly changing how HCV infection is managed clinically (26). SVR following anti-HCV therapy generally decreases the rate of HCC, supporting the importance of eradicating the virus to decrease HCC (27, 28). However, HCC can still form even after achieving SVR in subjects with pretreatment cirrhosis, elevated alanine aminotransferase (ALT) and steatosis, or prior HCC diagnosis, necessitating continued screening for HCC and development of therapy targeting cancer formation (29–31). In addition, as mentioned, many individuals with

chronic hepatitis C do not know that they are infected; by the time they are diagnosed, they have already progressed to an advanced disease state, which complicates antiviral therapy.

Development of various *in vitro* and *in vivo* HCV models has greatly increased our understanding of HCV and its mechanism of pathogenesis (32–35). Briefly, the HCV genome is 9.6 kb in length and contains one large open reading frame that codes for structural and nonstructural (NS) proteins. The open reading frame is flanked by untranslated regions (UTR) that function in viral gene expression and replication (Figure 1A). Structural genes code for core protein, which forms the viral capsid, and the envelope glycoproteins, E1 and E2. p7 and NS genes function in viral replication, polyprotein processing, and/or morphogenesis and viral release. HCV enters the cell through receptor-mediated endocytosis, which is facilitated by a series of molecular interactions that involve glycosaminoglycans (GAG), cluster of differentiation 81 (CD81), occludin (OCLN), claudin-1 (CLDN1), low-density lipoprotein receptors (LDLR), and scavenger receptor class B type I (SR-BI) (36) (Figure 1B). Upon entry, the positive-sense RNA genome is released in the cytoplasm, where translation of HCV RNA, initiated using an internal ribosomal entry site located in the 5' UTR, produces a large polyprotein. The polyprotein is then cleaved by host signal peptidase and NS2 and NS3/4A viral proteases to generate individual protein products, including NS5B, the viral replicase. The alternate reading frame of the core protein-coding sequence can also support the production of frameshift/alternate reading frame protein (F/ARFP), among others (37, 38). Then, NS5B and various proteins that comprise the replication complex replicate the genome through generation of a negative strand RNA. The newly synthesized positive sense RNA genome is then encapsidated, and virions are exported through the host secretory pathway (Figure 1B). Detailed review of the HCV replication cycle may be found elsewhere (36).

Many studies have demonstrated that HCV is capable of inducing tumors *in vivo*. Transgenic mice that express HCV genotype 1 core or core plus E1 and E2 under the control of eukaryotic elongation factor 1 α , a cytomegalovirus promoter, or in the liver under the control of an HBV promoter, spontaneously develop hepatic tumors, including HCC (35, 39, 40). HCC also forms spontaneously in transgenic mice expressing the entire open reading frame of genotype 1b HCV in the liver, or only NS5A (41, 42). Other studies reported an increased tumor rate in HCV core or NS5A transgenic mice treated with CCl₄, ethanol, diethylnitrosamine (DEN)/Phenobarbital, or co-expressing HBV X protein (39, 43–45). Spontaneous hepatic tumors in HCV transgenic mouse models generally occurred in the absence of hepatitis or cirrhosis, pointing to the direct oncogenic effects of HCV (35, 46, 47). This conclusion was supported by observations that core, NS3, NS4A, NS4B, NS5A, or polyprotein expression also transformed cells *in vitro*, either alone or in cooperation with Ras (48–53). Likewise, different HCV proteins accelerated tumor growth in nude mice (51, 54, 55).

II. HCV induces oxidative stress

A. Iron overload and nitric oxide generation

Chronic hepatitis C is characterized by pronounced increases in lipid peroxidation products (46, 56–60). Iron overload, independent of hemochromatosis gene mutations, has been

reported among hepatitis C patients (56, 59, 61–63). Hepatocytes and liver macrophages act as a substantial reservoir of iron, where 20 – 30% is bound to ferritin and hemosiderin (64). Decreased expression of hepcidin, a negative regulator of iron absorption, by virus-induced oxidative stress and HCV F/ARFP, may underlie elevated iron deposition (65, 66). Furthermore, IFN therapy decreased 4-hydroxy-2-nonenal protein adducts and hepatic iron deposits in responders (59).

HCV also increases the generation of nitric oxide ($\cdot\text{NO}$), an RNS, by transcriptional up-regulation of inducible nitric oxide synthase (iNOS) through a mechanism involving c-Jun (39, 67). Increased levels of nitrotyrosine were detected in liver biopsies from patients with chronic viral hepatitis, including hepatitis C, that co-localized with iNOS (68). These findings suggest increased nitrative stress during natural HCV infection

B. Mitochondrial ROS

In addition, HCV increases ROS generation. Specifically, elevated levels of superoxide and/or H_2O_2 have been demonstrated with HCV gene expression or infectious HCV in hepatocytes *in vitro*, as well as in hepatitis C patient liver (34, 69–74). HCV infection has been associated with significant mitochondrial abnormalities, and the mitochondrial electron transport chain was identified as a source of ROS in various HCV protein-expressing cells (Figure 2A). HCV-associated increases in oxidative stress or ROS were mediated by mitochondrial Ca^{2+} uptake and could be inhibited by diphenyleneiodonium (DPI), determined by monitoring ethidium generation from dihydroethidium, dihydrodichlorocarboxyfluorescein (DCF) diacetate fluorescence in isolated mitochondria and hepatocytes, and MitoSox fluorescence assay, (46, 60, 69, 75–79). HCV core localized to the mitochondria where Ca^{2+} uptake increased through the action of a mitochondrial Ca^{2+} uniporter; this then sensitized the organelle to mitochondrial membrane permeability transition (MPT) and increased superoxide generation at complex I (60, 69, 76, 78, 79) (Figure 2A). Mitochondrial glutathione (GSH), NADPH, and ATP synthesis decreased in the presence of HCV proteins (69, 76). Additionally, HCV core decreased the levels of prohibitin, a mitochondrial protein chaperon, as well as cytochrome c oxidase activity (80). Japanese fulminant hepatitis-1 (JFH1) strain, a genomic replicon, and HCV core likewise sensitized mitochondria to membrane depolarization or MPT induction by thapsigargin, which elevates cytosolic Ca^{2+} concentration by triggering a passive release of Ca^{2+} from the endoplasmic reticulum (ER) (73, 78). Exogenous peroxide, which has been shown to elevate cytosolic calcium content in hepatocytes, produced similar effects as thapsigargin on mitochondria in the presence of HCV (73, 78, 81). A spontaneous decrease in mitochondrial membrane potential in Raji cells, which could be inhibited by Bcl2, *N*-acetylcysteine (NAC), and an inhibitor of iNOS, was also noted with HCV (77).

Indeed, mitochondrial Ca^{2+} loading can increase mitochondrial ROS generation in hepatocytes by augmenting NAD(P)H synthesis by Ca^{2+} -sensitive mitochondrial dehydrogenases (82–84). Furthermore, excessive Ca^{2+} accumulation in mitochondria may trigger MPT, leading to osmotic swelling, breakage of the outer membrane, and release of cytochrome c, that has been associated with increased mitochondrial ROS (69, 85). Other mechanisms whereby Ca^{2+} loading has been shown to accelerate mitochondrial ROS

generation involve nitric oxide production and cardiolipin peroxidation (84). In addition, HCV has been shown to induce ER stress (75). These findings suggest that HCV increases mitochondrial ROS generation secondary to the mobilization of intracellular Ca^{2+} , although the detailed mechanism awaits further investigation.

C. Phagocytic Nox2

The Nox family enzymes consist of Nox and Dual oxidase (Duox) proteins (Nox1, 2, 3, 4, and 5 and Duox 1 and 2) that catalyze the transfer of electrons from NADPH to O_2 to generate superoxide and H_2O_2 (88). Unlike many other sources of ROS, such as mitochondria that produce ROS as a byproduct of aerobic metabolism, the primary function of Nox/Duox proteins is superoxide and H_2O_2 generation. Among the Nox proteins, the best characterized is Nox2 of the immune cells (2). Upon stimulation, Nox2 complex consisting of gp91^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, Ras-related C3 botulinum toxin substrate (Rac), and p40^{phox}, assembles with translocation of the cytosolic components, p47^{phox}, p67^{phox}, Rac, and p40^{phox} (2) (Figure 2B). This translocation leads to the respiratory burst, characterized by increased oxygen consumption and direct production of superoxide.

HCV has been shown to activate the phagocytic Nox2 (89, 90). Incubating human monocytes isolated from healthy blood donors with recombinant HCV NS3 protein, but not core, NS4, or NS5 proteins, resulted in superoxide production (89). NS3-stimulated superoxide generation was associated with phosphorylation and translocation of p47^{phox} and required Ca^{2+} influx, tyrosine kinase, and stress-activated protein kinase 2 p38 (SAPK2/p38) activities. This transient increase in superoxide generation observed in human monocytes was followed by an inhibition of phorbol 12-myristate 13-acetate-stimulated respiratory burst by NS3 (89). NS3 also induced a prolonged generation of ROS in mononuclear and polymorphonuclear phagocytes, that was inhibited by DPI and histamine (89, 90), and HCV proteins induced a prolonged activation of Kupffer cells in HCV-infected liver (91). In addition, HCV peptide (C5A), derived from the NS5A membrane anchor domain, could function as a chemoattractant and activated human phagocytic leukocytes, leading to massive phagocyte infiltration (92). The activated phagocytes in turn triggered dysfunction and/or apoptosis of T cells and natural killer cells, suggesting the role of HCV-associated oxidative stress in immune dysfunction (89, 90). Recently, it was also shown that Nox2 could be involved in the myeloid-derived suppressor cell-dependent T cell suppression in HCV infected patients (93). The liver contains 80 % of the host mononuclear phagocytic system (94). These reports suggest a significant role of phagocytic Nox2 in hepatic oxidative stress in chronic hepatitis C (Figure 2A).

D. Hepatocyte Nox1 and Nox4

In addition, hepatocytes, which are the primary site of HCV replication, express Nox family proteins (95–98). Recently, HCV was shown to increase Nox1 and Nox4 mRNA and protein levels in hepatocytes (71, 72) (Figure 2A, 2B). Nox1 and Nox4 served as a prominent source of ROS in hepatocytes producing infectious HCV of genotypes 2a and 1b or expressing HCV proteins (71, 72). Dominant negative Nox4, containing a C-terminus deletion, Nox4 or Nox1 knockdown, and DPI significantly decreased HCV-induced superoxide and/or H_2O_2 production in hepatocytes, compared to controls (71, 72). In fact, DPI is an inhibitor of

flavoproteins that inhibits Nox enzymes as well as complex I of the electron transport chain (86, 87). Similar increases in p22^{phox}, p67^{phox}, NOXA1, and NOXA2 mRNAs were also found (72). Nox4 and Nox1 proteins and Nox activity were significantly elevated in HCV-infected human liver (72). Likewise, human hepatoma (Huh7) cells inoculated with genotype 3 HCV-infected patient serum showed an increase in Nox4 mRNA (99). HCV core protein was sufficient to increase Nox4 levels *in vitro* (71).

Nox4 is thought to be constitutively active and regulated at the level of gene expression (see (100) for a review). Transforming growth factor beta (TGFβ), a fibrogenic cytokine, upregulates Nox4 expression in hepatocytes and hepatic stellate cells (HSCs) (96–98, 101). HCV activated human and murine Nox4 promoter activity in hepatocytes; furthermore, HCV increased levels of TGFβ in these cells and induced Nox4 transcription through TGFβ (71, 72). Increased levels of TGFβ have also been detected in patients with chronic hepatitis C (71, 72, 102). HCV was shown to induce TGFβ transcription through a redox-sensitive mechanism involving nuclear factor kappa B (NFκB) in hepatocytes, and through NFκB, activator protein 1 (AP-1), specificity protein 1 (Sp1), and signal transducer and activator of transcription 3 (STAT3) in HSCs (103–105). Hepatocyte Nox4 induction by TGFβ likely involves sma and mad related family (SMAD) proteins, which were shown to mediate TGFβ-induced Nox4 elevation in myofibroblasts (106). While the mechanism of how HCV increases Nox1 is unknown, TGFβ-stimulated increases in hepatocyte Nox1 and Rac proteins, or activities, have been reported (96–98).

Nox4, like other Nox enzymes, is composed of six predicted transmembrane domains and an intracellular C-terminal region that contains FAD and NADPH binding sites (107) (Figures 2B and 2C). The C-terminal FAD and NADPH binding sites and intracellular B loop are essential for its catalytic activity. It has also been reported that the D loop allows proper interaction between Nox4 and p22^{phox}, and the E loop is involved in H₂O₂ generation by Nox4 (107, 108). Nox4 has been localized to the cytoplasm as well as the plasma membrane and nuclear or peri-nuclear regions in various cell types (109–115). Nox4 was detected in both the inner and outer membrane of hepatic cell nuclei by immunogold electron microscopy, where Nox4 nuclear inclusions were also observed (116). The nuclear/peri-nuclear localization of Nox4 also increased with HCV in hepatocytes (72), with corresponding increases in nuclear nitrotyrosine and Nox activity that could be decreased by Nox4 small interfering RNA and an inhibitor of nitric oxide synthase (72). A recent study by Anilkumar *et al.* also described an N-terminus truncated Nox4, lacking transmembrane domains, that localized to nucleolus and produced superoxide in an NADPH-dependent manner (100, 117, 118). The nature of Nox proteins' association with nuclear compartments and the specific functions of nuclear Nox4, however, remain to be uncovered. TGFβ, which mediated Nox4 elevation by HCV in hepatocytes (71, 72), could increase nuclear Nox4 protein in other cell types (110, 111). Therefore, nuclear or peri-nuclear Nox4 could represent a normal cellular process that is enhanced by HCV. Nuclear location of Nox1 and Nox2 has also been reported in other studies (115, 119, 120). Together, evidence points to Nox1, Nox4, and Nox2 as major endogenous sources of ROS responsible for the HCV- and immune cell-mediated oxidative stress in hepatitis C patients.

E. Cytochrome P450 enzymes and decreased antioxidant defense

Another potential source of oxidative stress in HCV-infected individuals, particularly during alcohol intake, includes the cytochrome P450 (CYP) family enzymes (121, 122) (Figure 2A). CYP2E1 is involved in the hepatic detoxification of ethanol that leads to ROS generation (123, 124). HCV has been suggested to increase the level of CYP2E1 and potentiate CYP2E1-dependent ROS production in response to ethanol (46, 121, 125, 126). A recent study also reported that sulfaphenazole, which inhibits CYP2C9, decreased HCV-induced ROS, associated with an elevated expression of c-Myc (127). Interestingly, ethanol and its metabolite, acetaldehyde, activated H₂O₂ and superoxide generation in rat HSCs, and raloxifene, a non-specific inhibitor of Nox, decreased ROS elevation by acetaldehyde (128).

In addition, HCV can blunt the host antioxidant defense (see (129) and (130) for reviews). For example, hepatic, blood, and lymphocytic GSH is depleted with HCV, along with decreases in plasma antioxidant potential, vitamins A, B, and E, zinc, and selenium levels (57) (131) (46, 63, 132, 133). Such decline in antioxidant defense mechanisms would increase susceptibility to redox-mediated damage. It has been suggested that HCV directly interferes with the upregulation of antioxidant defense that normally occurs in response to sublethal oxidative stress, for example, by delocalizing small Maf proteins from the nucleus to the viral replication complex in the ER (134, 135). While GSH is clearly suppressed in patients, however, the extent to which HCV interferes with upregulation of the antioxidant defense is unclear (72, 136, 137).

III. Oxidative stress and pathogenesis of HCC

A. Oxidative stress is a key mediator of HCC

Evidence strongly pointing to a redox mechanism of hepatocarcinogenesis comes from *in vivo* studies that altered the levels of antioxidants or antioxidant gene expression to examine their effects. Mice deficient in copper, zinc superoxide dismutase (CuZn SOD, *sod1*^{-/-}), which converts superoxide to H₂O₂, exhibited reduced lifespan and increased incidence of nodular hyperplasia and HCC (138). Liver-specific deletion of nuclear factor-erythroid-2 related transcription factor-1 (Nrf1), involved in the regulation of antioxidant and phase II enzyme gene expression, likewise increased lipid peroxidation, oxidative DNA damage, and spontaneous liver cancer that occurred in 100 % of both male and female Nrf1-knockout mice compared to 0 % in littermate controls (139). These studies suggested that oxidative stress could be sufficient to induce liver cancer.

In addition, a recent study that examined the mechanism of hepatocarcinogenesis by a well-known tumor promoter, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), found that TPA increased mitochondrial ROS and DCF fluorescence (140). The TPA-induced tumor cell invasion could be decreased by addition of NAC and by decreasing the expression of ROS modulator 1 (Romo1) (140). TPA-associated ROS elevation was also suppressed by myxothiazol, an inhibitor of electron transport at complex III, and by DPI. TPA induced matrix metalloproteinase (MMP) promoter activity that was suppressed by non-specific inhibitors of Nox proteins, such as DPI and apocynin. In the DEN model of HCC in rats, DEN increased the oxidation of DCF in isolated liver mitochondria as well as conjugated

dienes, while decreasing catalase and SOD levels in the liver; nanocapsulated curcumin protected animals from histopathologic changes induced by DEN (141). In a different HCC model where the loss of Toll-like receptor 2 (TLR2), a pattern-recognition receptor, promoted DEN-induced HCC, pretreating the animals with NAC attenuated carcinogenesis and HCC progression (142); here, the TLR2 deficiency was thought to enhance HCC formation by decreasing activation of NF κ B, a key mediator of cell survival and neoplastic progression in the liver (142, 143). Other studies found a suppression of DEN-induced liver damage or HCC by butylated hydroxyanisole (BHA) (144, 145). NAC also attenuated D-galactosamine/lipopolysaccharide (LPS)-stimulated liver injury (146).

Likewise, double-transgenic mice bearing liver-specific expression of TGF α and c-Myc developed HCC with elevated DCF fluorescence and decreased GSH in hepatocytes (147–149). Preneoplastic and neoplastic lesions from the c-Myc, TGF- κ and c-Myc/TGF- α transgenic mice contained increased levels of Nox subunits, p47^{phox}, p67^{phox}, and Rac1, 70 kilodalton heat shock protein, as well as heme oxygenase-1; vitamin E reduced cell proliferation, apoptosis, and chromosomal alterations, and produced a 65% reduction in tumor incidence in animals (148, 149). These effects were associated with decreased levels of Nox subunits and iNOS, in addition to changes in DCF fluorescence. 2-Acetylaminofluorene induced mitochondrial redox cycling and increased superoxide levels concomitant with Rac activation in human hepatoma cells (150, 151). It is interesting that aflatoxin, alcohol, and HBV and HCV infections, which are the major etiologic agents of liver cancer, are each associated with oxidative stress (124, 152, 153). These studies indicate that oxidative stress is a common pathogenic feature in diverse HCC models. Note, however, that DCF oxidation is not specific to any particular reactive species and can be affected by diverse molecules including, cytochrome c; thus, changes in DCF fluorescence should be interpreted with caution, particularly during apoptosis (154).

Several lines of evidences also support the role of oxidative/nitrosative stress in HCV-associated liver cancer. ROS, detected using an electron paramagnetic resonance spin probe, were positively correlated with histological disease activity in chronic hepatitis C patients (74). Iron depletion therapy reduced oxidative damage, improved ALT levels, reduced inflammation and progression of fibrosis, and decreased the risk of HCC among hepatitis C patients (133, 155–157). Conversely, increasing hepatic iron enhanced liver steatosis and HCC formation in transgenic mice expressing HCV polyprotein (47, 158). Mitoquinone and antioxidant formulations containing silymarin, ascorbic acid, lipoic acid, L-GSH, or α -tocopherol generally had favorable effects on HCV-associated liver damage in patients (130, 159–161). Furthermore, the addition of BHA in the drinking water of HCV core transgenic mice significantly decreased DEN/Phenobarbital-driven hepatic tumor formation (39).

Cancer is characterized by sustained proliferative signaling, resistance to cell death, replicative immortality, angiogenesis, and activation of invasion and metastasis, and the processes underlying these hallmarks involve genome instability and inflammation (162). Specific mechanisms whereby oxidative stress contributes to HCC are summarized in the following sections and Figures 3A and 3B.

B. Oxidative DNA damage and inhibition of DNA repair

Chemical mutagenesis of DNA, leading to the mutation of critical cellular genes, is likely to be a key mechanism whereby oxidative stress contributes to hepatocarcinogenesis (Figure 3A). DNA strand breaks and other DNA modifications occur when highly reactive species such as hydroxyl radical ($\text{HO}\cdot$) attack bases and the deoxyribosyl backbone of DNA (163, 164). Chemical mutagenesis is likely to be random, but can facilitate the development of cancer when critical cellular regulatory genes are affected, such as activation of a proto-oncogene. 8-Hydroxydeoxyguanosine (8-OHdG), a product of redox-mediated DNA damage, for example, can induce G:C to T:A transversions frequently observed in cancers. Hepatic 8-OHdG is increased in HCC and has been positively correlated with HCC grades (165, 166). In patients with HCC, high levels of 8-OHdG in non-cancerous regions at the time of hepatectomy have been associated with an increased risk of tumor recurrence (167).

Increased levels of 8-OHdG and double strand DNA breaks were also found in hepatitis C patients as well as various cell culture and animal models of HCV, suggesting significant alteration of the host redox status with ongoing DNA damage (56, 59, 61–63, 67, 77, 131–133, 168–170). Higher levels of 8-OHdG were observed with HCV compared to HBV (131). Mutations in p53, β -catenin, B-cell lymphoma-6, and immunoglobulin heavy chain genes were elevated in HCV-infected B cells and HCV-associated peripheral blood mononuclear cells, lymphomas, and HCC (67, 168). In addition, significant membrane alterations with loss of normal chromatin organization could be found in HCV transgenic hepatocytes (40). HCV core, the primary factor responsible for virus-induced changes in the host redox status, functioned as a tumor initiator in phenobarbital-treated mice (171).

Hydroxyl radical, which induces 8-OHdG and other DNA damage products, is produced by a non-enzymatic reaction between H_2O_2 and ferrous iron (Fe^{2+}), referred to as the Fenton reaction (Figure 2A). Because of its high reactivity and propensity to react with whatever molecule near the site of its generation, hydroxyl radical needs to be generated in close proximity to DNA to directly induce DNA damage. HCV-induced DNA damage was reduced by NAC, as well as strategies that decreased iron or the production of nitric oxide (77). 8-OHdG decreased with iron reduction therapy in hepatitis C patients and increased with iron overload in HCV transgenic mice (47, 133). These findings suggest that excess iron and sources of superoxide/ H_2O_2 and nitric oxide (see section II) all contribute to genotoxicity during HCV infection via hydroxyl radical and peroxynitrite formation (Figure 2A). The discovery that Nox4 is present in or around the nucleus of hepatocytes actively replicating HCV is intriguing as it would increase the probability of DNA damage (72) (Figure 2A). For example, a shift in ROS generation from the cytosol to the nucleus accelerated DNA damage in peroxiredoxin 1-knockout mice (172). Nucleolar localization of the 28kD Nox4 splice variant was associated with increased DNA damage, as determined by phosphorylated histone family H2A, member X (H2AX).

HCV also interferes with DNA repair, which would exacerbate its mutagenic effects. 8-OHdG is eliminated mainly through base excision repair initiated by 8-oxoguanine glycosylase (OGG1), a DNA glycosylase. Human OGG1 polymorphism was associated with some cancers and could predict survival in HCC patients (173, 174). A positive correlation was observed between 8-OHdG levels, disease stage, telomerase activity, OGG1

polymorphisms and ALT/gamma-glutamyl transpeptidase levels in HCC patients (175). OGG1 activity could be modulated by reversible cysteine modification and inhibited by nitric oxide; the latter was associated with the formation of S-nitrosothiol adducts and loss or ejection of zinc ions (176, 177). Recently, hXRCC3, a different DNA repair protein that participates in homologous recombination, was also suggested to be redox-regulated (178). HCV was found to induce error-prone DNA polymerases while decreasing DNA glycosylase activity in an iNOS-dependent manner (39, 168). HCV also interfered with nonhomologous end-joining repair. Interestingly, HCV core interacted with 14-3-3 protein (179). H2AX, involved in DNA damage response and repair, was suggested to affect Nox1 activity indirectly by binding to 14-3-3 zeta in HCC cells (180). Over-expression of H2AX triggered dissociation of 14-3-3 zeta from NoxA1, leading to Nox1 activation and induction of oxidative stress (181, 182).

C. Redox regulation of epigenetics in HCC

The pathogenesis of HCC involves epigenetic as well as genetic modifications. Previously, H₂O₂ was shown to decrease the expression of E-cadherin through epigenetic silencing (166). Loss of E-cadherin, a cell adhesion molecule that regulates epithelial-mesenchymal transition (EMT), occurs in metastasis and is associated with poor prognosis of HCC. Exogenous H₂O₂ and menadione, a redox-cycling quinone, increased hypermethylation of the E-cadherin promoter by histone deacetylase 1 and DNA (cytosine-5)-methyltransferase 1 (DNMT) through increased expression of snail, which silenced the expression of E-cadherin (166, 183). CuZn SOD, catalase, and GSH peroxidase 1 activities also declined with HCC grade, and prolonged exposure to H₂O₂ increased methylation of the catalase promoter, downregulating its transcription in HCC cell lines (166, 184).

Decreased expression of E-cadherin occurred as an early marker of HCC in HCV-related cirrhosis (185). HCV triggered EMT, with loss of E-cadherin, and elevated snail and twist in primary human hepatocytes and in HCV-infected liver biopsies (186). Hypermethylation of the E-cadherin promoter, and increased the expression of DNA (cytosine-5)-methyltransferase 1 and 3 by HCV core have also been described (187, 188). In a recent study, HCV increased DNA methylation in severe combined immunodeficient (SCID) mice carrying a urokinase-type plasminogen activator transgene controlled by an albumin promoter (or, uPA/SCID mice), where the liver was repopulated with human hepatocytes (189). Changes in DNA methylation were accompanied by increased dihydroethidium stain and induction of IFN γ (189).

D. Inflammation, HCC, and Nox enzymes

Currently, there is no established HCV small animal model that shows spontaneous liver tumors, as well as chronic hepatitis and hepatic fibrosis, which has limited our understanding of the role that inflammation plays in HCV-induced pathogenesis. For example, a recently described chimeric mouse model with a humanized liver and reconstituted T cell immunity can be infected by HCV and show signs of inflammation, hepatitis, and fibrosis, but whether hepatic tumors form in these animals is unknown (190). In addition, transgenic mice that express human HCV receptors CD81, OCLN, CLDN1, and SRB-1, referred to as four HCV entry factors, were developed. These mice have complete

host immunity and can be infected with HCV and show increases in IFN γ , tumor necrosis factor alpha (TNF α), and IFN stimulated genes, but whether these mice show natural disease progression is unclear (191).

Nevertheless, emerging data from various *in vitro*, animal, and clinical studies suggest an important role of inflammation in HCC. Indeed, although spontaneous tumors/HCC can form without hepatitis in several HCV transgenic animal models (46, 47), natural HCV infection is marked by chronic hepatitis (90–92, 192–194). Kupffer cells, which are resident liver macrophages, represent 15 to 20% of the total liver cell population. Studies indicate that Kupffer cells/Nox2 are activated in chronic hepatitis C (91, 92, 192, 195) (also, refer to section IIC) (Figure 3B). In addition, myeloperoxidase genotype was associated with the progression of hepatic fibrosis and HCC in individuals with chronic hepatitis C, suggesting the involvement of neutrophils in pathogenesis (196, 197). Peripheral blood neutrophils from children with chronic hepatitis C demonstrated an increased expression of TLR2 and TLR4 that was associated with increased ALT and intensified hepatic necrosis (198). Rises in malondialdehyde and protein carbonyls, and decreases in GSH and sulfhydryl proteins in the plasma preceded ALT flare-ups in hepatitis C patients, supporting a role of oxidative stress in inflammation and tissue damage (199).

• **Lymphotoxin and NF κ B**—Recently, a dramatic upregulation in lymphotoxin- β levels was identified in tumors from HCV transgenic mice, which was accompanied by activation of NF- κ B, elevated chemokines, and intra-tumoral recruitment of mononuclear cells (200) (Figure 3B). Inactivation of IKK β reduced the tumor rate in these animals (200). Previously, liver-specific expression of lymphotoxin was shown to trigger liver inflammation and HCC in mice, and the study supported the importance of NF κ B in HCC (201, 202). NF κ B, a chief regulator of pro-inflammatory responses and apoptosis, is regulated in the cytoplasm as well as nucleus by redox mechanisms (203). The mechanism of NF κ B activation in the cytoplasm involves protein kinase A-mediated serine phosphorylation as well as H₂O₂-induced disulfide bond formation in NEMO that inhibits the I κ B kinase complex (204, 205). Cysteine oxidation of the p50 subunit, on the other hand, inhibits DNA binding of NF κ B; thus, NF κ B requires reduction by redox factor 1 (Ref-1) in order to function (206, 207). For example, Nox2 and Nox1 activated c-Src and NF κ B and increased hepatic production of TNF α during ischemia/reperfusion (208).

• **Pro-inflammatory cytokines**—Serum cytokines, such as TNF α and interleukin-1 beta (IL-1 β), are elevated in chronic hepatitis C (121, 209, 210) (Figure 3B). Kupffer cells acted as the main source of IL-1 β during HCV infection (211). These cells are also likely to be a major source of TNF α in chronic hepatitis C (91, 121). Increased levels of IL-1/IL-1 β , IL-6, and TNF α were also demonstrated in HCV core transgenic mice that developed liver tumors (39, 212). The mitogen-activated protein kinase (MAPK) pathway is a well known target of redox signaling (213). c-Jun N-terminal kinase (JNK) and AP-1 were activated in the core transgenic mouse liver (39, 214), and cytokine elevation was found to be mediated by c-Jun and STAT3; genetic deletion of c-Jun and STAT3 decreased tumor rates in these mice (39) (Figure 3B). HCV NS5A-induced activation of JNK, p38 MAPK, and AP-1 could also be

inhibited by NAC and dithiothreitol (215, 216). NAC and pyrrolidine dithiocarbamate (PDTC) also affected NF κ B and STAT3 activation by HCV (75).

Secretion of IL-1 β requires inflammasome-mediated activation of caspase 1 (217). HCV increased the secretion of IL-1 β by activating NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasomes in Kupffer cells, as well as human hepatoma cells (136, 211) (Figure 3B). While NALP3 inflammasome/caspase 1 activation has been suggested to require ROS and to regulate Nox2 under certain conditions, HCV-stimulated IL-1 β secretion in hepatocytes could be blocked by PDTC (136, 217, 218). These studies suggest that NF κ B and cytokine modulation by HCV could be redox-mediated.

• **DEN-induced liver damage and p47^{phox}**—Previously, inflammation, apoptosis, and regenerative DNA synthesis subsequent to DEN-induced liver damage were shown to be diminished in mice deficient in p47^{phox} (219). DEN directly activated Kupffer cells to produce superoxide and TNF α , and these responses were attenuated in the p47^{phox} knockout mice. Furthermore, DEN induced DNA damage in hepatocytes *in vivo*, but the DNA damage was reduced in p47^{phox}-deficient animals. These studies suggested that superoxide generation by phagocytes during inflammation/liver injury produces genotoxic effects in hepatocytes, which may contribute to tumor initiation and promotion (219).

• **TLR4 and HCC**—TLR4, a pattern recognition receptor, is activated by endotoxins such as LPS present in gram negative bacteria. Alcohol is a major cofactor in HCV-associated liver disease (220). In alcohol-induced endotoxemia, gut permeability is increased, resulting in bacterial translocation and activation of TLR4. Nox2/p47^{phox} is a key mediator of LPS/TLR4-stimulated ROS generation in polymorphonuclear cells and macrophages (221–224) (Figure 3B). Early effects of alcohol on liver injury also involve LPS-stimulation of Kupffer cells that serve as the predominant source of TNF α (225). The p47^{phox} knockout mice failed to show an increase in free radical detection in bile by electron spin resonance, activation of NF κ B, increase in TNF α mRNA, or liver pathology in response to alcohol treatment (225). Also, ethanol and LPS activated Rac1-GTPase and translocation of p67^{phox} in rat Kupffer cells (226). TLR4 stimulation activated Rac1 through IRAK-1 in macrophages (227).

Recent studies indicate that TLR4 is a key factor in HCC formation; TLR4 increased cell proliferation in response to carcinogens, and depleting the intestinal microbiota decreased the incidence of HCC (145, 228). TLR4 is also important in HCV-induced HCC. For example, HCV can also up-regulate TLR4 (229); TLR4, LPS, as well as C-X-C motif chemokine 10 (CXCL10), an endogenous chemokine that activates TLR4, are likewise elevated in hepatitis C patients (230–233), although a downregulation of TLR4 and associated apoptosis was also reported in one study (234). A recent study further indicated that alcohol and LPS treatments synergized with HCV NS5A to enhance the rate of hepatic tumors through a progenitor cell marker, Nanog (44) (Figure 3B). LPS increased tumorigenesis in p53-deficient and TLR4-transduced hepatic progenitor cells, and enhanced the proliferation of tumor initiating cells (39, 235). Intestinal colonization by the gram negative bacteria, *H. hepaticus*, promoted aflatoxin-and HCV transgene-induced HCC in mice, that was associated with increased hepatocyte turnover, Wnt/ β -catenin activation, oxidative injury, and activation of NF κ B-regulated networks in the lower bowel and liver

(236). Clinically, the rs4986791 T allele of TLR4 was associated with a decreased risk of HCC in chronic hepatitis C patients (237). Concanavalin A-induced liver injury and cell death were also attenuated by antagonism of CXCL10 (232). These studies suggest that Nox2 could be a key mediator of hepatocarcinogenesis mediated by the TLR4 pathway during HCV infection, and other conditions leading to HCC. A recent study reported that macrophages from p47^{phox} knockout mice showed greater NFκB activation by LPS than control mice (238); although it is not clear why p47^{phox} exhibited different effects on NFκB in the study, but the results are consistent with the regulation of NFκB in the nucleus by Ref-1 (203).

LPS stimulation was also shown to up-regulate Nox1 transcription in bone marrow-derived macrophages; here, Nox1 transcription was associated with activation of IRAK-1 and increased recruitment of p65 and C/EBP to the Nox1 promoter (227). LPS-stimulated Nox1 expression has also been associated with accumulation and nuclear translocation of β-catenin in macrophages (239). In addition, TLR4 signaling required Nox4 in the Raw264.7 murine macrophage cell line, HEK293 cells, and human aortic endothelial cells (240–242). Whether Nox proteins are involved in TLR4 signaling in hepatocytes, however, is unknown.

• **TNFα and hepatocyte proliferation**—Increased expression of pro-inflammatory cytokines such as TNFα is associated with apoptosis but has also been shown to be essential for hepatocyte proliferation and liver regeneration (243–245). TNFα increased cell-cycle progression and 8-oxo-dG levels in murine hepatocytes without stabilizing p53 (246, 247). TNFα also up-regulated hypoxia-inducible factor 1-mediated Forkhead box M1 (FoxM1) expression that could be inhibited by BHA; these changes were associated with HCC proliferation and resistance to apoptosis (245). FoxM1 is a cell cycle regulator that is upregulated in human HCC tissues, and FoxM1 expression has been associated with HCC formation in mice (248–250). Furthermore, TNFα treatment of hepatocytes increased mitochondrial ROS generation at complex I and complex III that in turn, activated NF-κB and increased cell migration (251) (Figure 2). TNFα also up-regulated Nox2, Nox4, Nox1, p67^{phox}, p47^{phox}, p22^{phox}, and/or Duox2 gene expression in other systems (252–254). Nox1 and Nox4, which are elevated by HCV (71, 72), also contain conserved gamma activated sequence elements in their promoters, and were activated by IFNγ in human large intestinal epithelial cells and aortic smooth muscle cells; likewise, Duox2 was activated by IFNγ in primary respiratory tract epithelial cell cultures (255–257). IFNγ levels are elevated in the liver and serum in the presence of HCV (189, 191, 210). Whether Nox proteins are regulated by TNFα and IFNγ and mediate their effects in hepatocytes, remains to be tested.

E. Hepatic fibrosis, TGFβ, and Nox enzymes

Liver fibrosis is characterized by accumulation of collagen and other extracellular matrix components in response to chronic liver injury (258). Cirrhosis is an end-stage process of fibrotic liver degeneration (259). HSCs and Kupffer cells are major fibrogenic cell types in the liver. During hepatic fibrosis, quiescent HSCs transdifferentiate to myofibroblasts and produce cytokines, extracellular matrix proteins, and glycoproteins (260). Oxidative stress is a key mediator of hepatic fibrosis, and ROS and lipid peroxidation can stimulate the production of type I collagen (258, 259, 261, 262). Increased iron levels have also been

shown to induce inflammatory cytokines and subsequent activation of HSCs, leading to fibrosis (263). A majority of HCV-associated HCC cases are preceded by cirrhosis (21, 264). Thus, the induction of hepatic fibrosis could be another process through which oxidative stress facilitates HCC formation (Figure 3A).

Accumulating data indicate the importance of Nox2, Nox1, and Nox4 proteins in hepatic fibrosis. Nox2 and Nox1 were upregulated, in addition to Nox4, in activated HSCs (265). Bile duct ligation (BDL)-induced liver fibrosis was attenuated in p47^{phox} knockout mice compared to controls, where Nox1 and Nox2 were shown to play a role (265–267). Also, phagocytosis of apoptotic hepatocytes activated HSCs and collagen production through Nox2 activation (268, 269). CCl₄-induced liver fibrosis and hydroxyproline were similarly reduced in gp91^{phox} knockout mice (270). Furthermore, pharmacological inhibition of Nox1 and Nox4 with GKT137831 significantly reduced liver fibrosis induced by bile duct ligation or CCl₄ (271, 272). Fibrogenic responses to angiotensin II were blunted by NAC and DPI treatments, and angiotensin II-stimulated DNA synthesis and cell migration of HSCs were reduced in p47^{phox} knockout mice (266). On the other hand, decreased functions of neutrophils, including respiratory burst, are commonly observed in decompensated patients with cirrhosis (273).

TGF β , which activated Nox4 and Nox1/Rac in hepatocytes (71, 72, 96–98), is a major factor accelerating the progression of organ fibrosis (274). TGF β stimulates type I collagen transcription and regulates the expression of MMPs and their inhibitors (274). The fibrogenic effects of TGF β are mediated by ROS (259). Nox4 was also increased by TGF β in fibroblasts, and Nox4 mediated TGF β functions in hepatocytes, fibroblasts, as well as HSCs (106, 111, 271, 275, 276). The discovery that HCV induces Nox4 in a TGF β -dependent manner, therefore, is potentially significant and suggests a role of hepatocyte Nox4 and oxidative stress in hepatitis C-associated fibrosis. Increased expression of Nox4 was found in HCV-infected patient liver, which increased with fibrosis degree (72, 101). Duox1, Duox2, and Nox4 were expressed at higher frequencies in tumor specimens from patients (277). Furthermore, treatment with DPI abrogated changes in fibrogenic gene expression induced by HCV and human immunodeficiency virus in HSCs and hepatoma cells (278). HSC activation by hepatocyte apoptotic bodies, which is likely Nox2-dependent (269), was also found to be amplified when the apoptotic bodies contained HCV nonstructural proteins (279). Supplemental treatment of chronic hepatitis C patients with CuZn SOD reduced TGF- β mediated liver fibrosis (280). Other enhancers of fibrosis, increased in response to HCV, include other growth factors, chemokines, and cytokines, such as TNF- α (258, 281).

TGF β , on the other hand, has a tumor suppressive effect on hepatocytes. A recent study showed that Nox4 expression was significantly downregulated during active hepatocyte proliferation during DEN-induced hepatocarcinogenesis, and after partial hepatectomy in mice (282). In the same study, silencing of Nox4 was associated with increased proliferation of liver tumors and untransformed cells as well as decreased activation of caspase 3 by TGF β . These results are consistent with previous studies that reported a role of Nox4 in TGF β -induced senescence arrest/apoptosis in hepatocytes (276, 283–285). However, loss of the tumor suppressive function of TGF β is frequently observed in cancers (274). In chronic

HCV infection, an inflammatory environment, mediated by JNK-activated IL1 β , was also suggested to shift the tumor-suppressive signaling of TGF β to a migratory and mesenchymal phenotype associated with tumor progression (286). HCV itself suppressed TGF β -induced apoptosis (287, 288). TLR4 signaling also interfered with the tumor suppressive effects of TGF β in tumor-initiating cells through Yap1, and the TGF β pathway in turn suppressed TLR4 signaling (235) (Figure 3B). In addition, TGF β treatment of rat pluripotent liver progenitor cell (LPC)-like cells impaired the LPC potential but conferred tumor initiating cell properties (289). TGF β level was positively correlated with LPC and tumor initiating cell markers in rat models of HCC as well as cirrhotic livers of human HCC patients (289). Furthermore, VAS2870, a Nox inhibitor, decreased serum-dependent growth and phosphorylation of AKT and ERK and enhanced TGF β -induced apoptosis in HCC cell lines (290). Nox1, which was co-elevated with Nox4 by HCV (71, 72), was also suggested to provide a partial resistance to hepatocyte apoptosis induced by TGF β (290, 291), and excessive stimulation of the MAPK/ERK pathway produced similar effects (284). Furthermore, NS5A was recently found to interact with mixed lineage kinase 3 that activated p38 MAPK to combat apoptosis induced by sulfhydryl oxidizing agent, dithiodipyridine (292). HCV core protein suppressed cellular senescence stimulated by H₂O₂ by downregulating p16 (293). Mutation or allelic loss of caspase-8, an initiator caspase, is also frequently observed in HCC (294). In advanced HCC associated with chronic hepatitis C, apoptosis was decreased with downregulation of Ras (295). On the other hand, recent studies using HCV infection models suggest that HCV is pro-apoptotic (70, 296–298). Thus, HCV most likely regulates apoptosis differently at various stages of pathogenesis, and the role of hepatocyte Nox proteins in the regulation of cell apoptosis and proliferation should be further defined.

F. Hepatic steatosis and other mechanisms

The incidence of hepatic steatosis, observed in almost half of the HCV infected patients (1, 299), strongly correlates with the development of liver fibrosis, cirrhosis and eventually HCC (300–302). Various *in vivo* studies show that HCV core and NS5A are associated with steatosis (303–306), and HCV core, NS2 and NS4B proteins also induced accumulation of lipid droplets *in vitro* (307–309).

HCV can upregulate sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferators-activated receptor (PPAR) γ , which in turn increase hepatic fatty acid synthesis (310). PPAR α and proteasome activator 28 γ were necessary for the induction of steatosis as well as hepatocarcinogenesis in HCV core transgenic mice (304, 305). Activation of SREBP-1 by HCV was sensitive to PDTC, Ca²⁺ chelator, and an inhibitor of phosphoinositide 3-kinase (PI3K) (311). Other metabolic factors such as insulin resistance and hyperglycemia associated with HCV may also contribute to lipid accumulation indirectly. Thus, the increased accumulation of polyunsaturated fatty acids in steatosis resulting from the combination of viral and metabolic factors, together with the increased hepatic iron reserves detected in the liver of chronic hepatitis C patients may make the steatotic liver an easy target for lipid peroxidation (312, 313) (see II-A). Lipid peroxidation can result in the formation of DNA adducts (314).

HCV also modulates regulators of cell proliferation and cell death that include retinoblastoma protein (pRb), p53, Wnt/ β -catenin, and MAPK pathways (see (315–317) for reviews). pRb is a G1/S regulator of the cell cycle that functions by inhibiting E2F-dependent transcription. p53 is a tumor suppressor protein that functions in cell cycle regulation, induction of apoptosis, and DNA repair in the presence of DNA damage. Aberrant activation of the Wnt signaling pathway stabilizes β -catenin allowing entrance into the nucleus to activate transcription of Wnt-target genes that can mediate hepatocarcinogenesis (318). HCV can decrease the level of pRb and p53 and interact with these factors (315–317). HCV also up-regulated β -catenin-mediated transcription *via* PI3K (319), which may contribute to HCC by increasing hepatocyte proliferation, tumorigenesis, and EMT (127, 320, 321). Several studies reported that some of these effects of HCV could be inhibited by NAC or PDTTC, including PI3K and osteopontin that regulated β -catenin (307, 321, 322).

Moreover, ROS and Nox proteins have been shown to mediate angiogenesis (323, 324) (325), but whether Nox enzymes mediate angiogenesis in HCV-induced HCC remains to be tested. H₂O₂ could increase iron uptake in murine fibroblasts (326, 327), and it would be interesting to test whether H₂O₂ contributes to hepatic iron accumulation in chronic hepatitis C. Another potential mechanism of hepatocarcinogenesis involves increased survival of the infected cells through activation of Nrf2 by reactive species, although an attenuation of Nrf2 and cellular antioxidant response by HCV has also been reported (134, 135, 328, 329) (see IIE).

IV. Oxidative stress and HCC – conclusions and considerations for therapy

Chronic hepatitis C is a global health concern. Accumulating evidence suggests that oxidative stress plays an important role in HCC induction by HCV as well as other conditions leading to HCC. Cancer is a multi-step process, and the mutagenic effects of HCV-associated oxidative stress are likely to cooperate with other pathogenic mechanisms of HCV, such as inflammation, to induce HCC (Figure 3). These mechanisms may include redox signaling as well as oxidative damage (Figure 2A).

The multikinase inhibitor, sorafenib, was approved for therapeutic use in advanced, non-resectable HCC (330). Treatment with sorafenib could improve the one-year survival rate to 44% *versus* 33% in the control group, and lengthened time to radiological progression to 5.5 months *versus* 2.8 months in the control group (330). However, the limited success, growing increase in HCC cases, as well as reports of serious adverse events resulting in treatment discontinuation, warrant the investigation of adjunct therapies to enhance treatment efficacy. *In vitro* and *in vivo* studies combining sorafenib with mammalian target of rapamycin inhibitors suggest enhanced benefit over monotherapy (331). Indeed, HCC is heterogeneous, and the pathogenesis likely involves multiple mechanisms, calling for continuation of basic and clinical studies aimed at investigating the therapeutic benefit of multiple drug combinations in the treatment of HCC, in addition to development of an HCV vaccine.

The common occurrence of increased reactive species in diverse HCC models suggests that strategies designed to reduce the levels of reactive species could be beneficial in preventing

HCC. Antioxidant strategies generally produced favorable effects in various *in vitro* and *in vivo* models of HCC and in HCV-associated liver damage/HCC, although data on human HCC cases are still limited (130). A recent *in vitro* study further suggested that oxidative stress may contribute to the high baseline sequence variability of HCV that complicates antiviral therapy (332–336); iron chelator and GSH ester could decrease HCV sequence variability (332). In clinical studies, some antioxidant strategies enhanced the virological outcome of antiviral therapy in patients whereas other studies showed either no effect or inconsistent benefit (130); possible explanations include limited bioavailability, efficacy, and non-specific effects of traditional antioxidants. Altogether, these studies suggest that combining antioxidant strategies with antivirals directly targeting HCV may also improve antiviral efficacy. In this regard, Nox proteins can be inhibited pharmacologically, and this may be more effective at suppressing ROS levels than blunting the damage once reactive species are already generated. Additional studies, however, will be needed to determine whether strategies that target oxidative stress would be beneficial in the treatment of HCC and HCV infection. Possible effects on antiviral host immunity will also need to be evaluated. Recent development of specific Nox-deleted mice and mouse models that support complete HCV replication with partial, or complete adaptive immunity, provide new tools to test these hypotheses (190, 191, 324). Finally, tipping the metabolic balance further towards greater oxidative stress has been associated with cancer cell death, and has been suggested to aid in cancer treatment once cancer has established (337). Thus, the possibility of increasing oxidative stress to enhance the destruction of HCC presents a different perspective that may, too, need to be considered.

Acknowledgments

This work was supported by NIH grant R01CA158271 to J.C.

Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
8-OHG	8-hydroxyguanine
ALT	alanine aminotransferase
AP-1	activator protein 1
BHA	butylated hydroxyanisole
CD81	cluster of differentiation 81
CXCL10	C-X-C motif chemokine 10
CYP	cytochrome P450
DCF	dihydrodichlorocarboxyfluorescein
DEN	diethylnitrosamine
DPI	diphenyleneiodonium
DUOX	dual oxidase

EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
F/ARFP	frameshift/alternate reading frame protein
FoxM1	Forkhead box M1
GAG	glycosaminoglycans
GSH	glutathione
H2AX	H2A histone family, member X
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HSCs	Hepatic stellate cells
Huh7	human hepatoma
IFN	interferon
IL-1β	interleukin-1 beta
iNOS	inducible nitric oxide synthase
JFH1	Japanese fulminant hepatitis-1
LDLR	low density lipoprotein receptor
LPC	liver progenitor cell
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MPT	membrane permeability transition
NAC	<i>N</i> -acetylcysteine
NALP3	NACHT, LRR and PYD domains-containing protein 3
NFκB	nuclear factor kappa B
Nox	NADPH oxidase
Nrf	nuclear factor-erythroid-2 related transcription factor
NS	nonstructural
OCLN	occludin
OGG1	8-oxoguanine glycosylase
PDTC	pyrrolidine dithiocarbamate
PI3K	phosphoinositide 3-kinase

PPAR	peroxisome proliferator-activated receptor
pRb	retinoblastoma protein
Rac	ras-related C3 botulinum toxin substrate
RNS	reactive nitrogen species
Romo1	ROS modulator 1
ROS	reactive oxygen species
SCID	severe combined immunodeficient
SMAD	sma and mad related family
SOD	superoxide dismutase
Sp1	specificity protein 1
SR-B1	scavenger receptor class B type I
STAT	signal transducer and activator of transcription
SVR	sustained virological response
TGF	transforming growth factor
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
UTR	untranslated region

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Fig. 1. HCV genome and replication cycle

(A) Genome organization of HCV. SP indicate serine protease. (B) Replication cycle of HCV.

Fig. 2. Mechanisms of HCV-induced oxidative/nitrosative stress and hepatic Nox proteins
 (A) Sources of ROS and RNS during HCV infection. HCV increases superoxide ($O_2^{\cdot-}$) and H_2O_2 formation in hepatocytes by Nox enzymes, Complex I of mitochondria, and CYP enzymes in combination with alcohol. Superoxide generation by phagocytic Nox2 from activated Kupffer cells and decreased antioxidant defense will exacerbate oxidative stress. Increased nitric oxide ($\cdot NO$) generation through iNOS and subsequent formation of peroxynitrite ($ONOO^-$) from nitric oxide and superoxide, and hydroxyl radical ($HO\cdot$) from H_2O_2 and Fe^{2+} are also shown. Oxidative stress will contribute to pathogenesis of HCC by DNA, lipid, and protein damage as well as modification of cell signaling. (B) Schematics of Nox1, Nox2, and Nox4 protein complexes. (C) Nox4 protein domain map. FAD and NADPH binding sites are marked.

B

Fig. 3. Oxidative stress in the pathogenesis of HCC

(A) Oxidative stress in the pathogenesis of HCV-associated HCC. A majority of HCV infection results in chronic infection that can progress to cirrhosis in 10 – 30 years. HCC forms in 4 – 7% of chronic hepatitis C patients with cirrhosis per year (see I-B). HCV induces oxidative stress and chronic inflammation that can lead to liver tumors/HCC through DNA damage, increased cell proliferation, regulation of cell death, and fibrosis that increases the risk for HCC. (B) Inflammation-driven pathways to HCC in chronic hepatitis C. Some of the main events contributing to HCV-induced HCC involving inflammation and redox-active processes are shown. (i) HCV activates Nox2 of monocytes/Kupffer cells and polymorphonuclear cells (PMN). (ii) Lymphotoxin β contributes to HCC formation in an NF κ B-dependent manner. AP-1 and Stat3 also mediate cytokine elevation. Activation of NALP3 inflammasome in hepatocytes and Kupffer cells by HCV contributes to increased IL-1 β secretion. (iii) TLR4-mediated inflammation and liver injury mediated by Kupffer cells require p47^{phox}, a component of Nox2. (iv) TLR4 stimulation by LPS, CXCL10, and alcohol mediates transformation of hepatic progenitor cells and increases proliferation of tumor-initiating cells through Yap, while inhibiting tumor suppressive effects of TGF β .