Interleukin-1 Inhibits Hepatitis C Virus Subgenomic RNA Replication by Activation of Extracellular Regulated Kinase Pathway

Haizhen Zhu and Chen Liu*

Department of Pathology, Immunology, and Laboratory Medicine, University of Florida
College of Medicine, Gainesville, Florida 32610-0275

Received 17 December 2002/Accepted 12 February 2003

Interleukin-1 (IL-1) plays an important role in the inflammatory process. Some studies have demonstrated that IL-1 production was impaired in patients with chronic infections of hepatitis C virus (HCV), implying that IL-1 may play a role in viral clearance. Using an HCV subgenomic replicon cell line, we demonstrate that IL-1 can effectively inhibit HCV subgenomic RNA replication and viral protein expression, suggesting that IL-1 has direct antiviral activity. The inhibitory effect is associated with the extracellular regulatory kinase (ERK) activation. In addition, we also show that IL-1 can induce one of the interferon-stimulated genes (ISGs), 1-8U, which exhibits antiviral activity. However, it has no effect on the other ISG, 6-16, suggesting that IL-1 induces novel antiviral pathways within a cell.

The hepatitis C virus (HCV) is a plus-strand RNA virus that causes acute or chronic hepatitis, cirrhosis, and even hepato-cellular carcinoma (20). The present treatment is alpha interferon (IFN-α)-based monotherapy or combination therapy with ribavirin. The overall response rate to the combination therapy is approximately 50 to 60%, with a lower response rate for HCV genotype 1 infection, the predominant genotype in Western countries (6, 22). One of the major clinical issues has been to manage patients who do not respond to IFN treatment. Although the viral molecular structure has been characterized (4, 16), the underlying pathogenesis of HCV infection and the detailed molecular mechanisms for IFN-α action are still not clear.

The histology of infected livers is characterized by inflammatory cell infiltration (predominantly lymphocytes), hepatocyte apoptosis, and various degrees of fibrosis (11). The inflammation caused by viral infection in the liver plays the central role in the pathogenesis, although the regulatory mechanisms are not fully defined. It is generally believed that the severity and extent of tissue inflammation are initiated and maintained by numerous cytokines. These cytokines are secreted by many types of inflammatory cells, and the cytokines themselves are also responsible for the migration and activation of inflammatory cells. Interleukin-1 (IL-1) is one of the most prominent proinflammatory cytokines involved in tissue inflammation (8). The key role of IL-1 appears to be related to its function concerning inflammatory cells, which is crucial for viral clearance and the host immune response. IL-1 has two isoforms, IL-1α and IL-1β, and both isoforms have similar biological functions. IL-1 can exert numerous biological effects on multiple cell types. The effect of IL-1 on noninflammatory cells, such as virus-infected cells, is still not clear.

Changes in IL-1 expression in HCV-infected patients have been well documented. Most studies showed that chronically infected patients had somewhat lower levels of spontaneous and stimulated IL-1 expression by the peripheral blood mononuclear cells (9, 23–25), although some other studies have suggested that patients with HCV had increased serum IL-1 levels (15). Moreover, Daniels et al. have shown that the resolution of the hepatitis B virus (HBV) infection during IFN-α treatment was accompanied by increasing production of IL-1, suggesting that IL-1 production is associated with the IFN therapy response rate (5). The precise biological roles of IL-1 in HCV infection are not defined. It was proposed that IL-1 could help viral clearance by regulating immune response, in particular, the antigen presentation process. In addition, molecular analysis has shown that IL-1 can enhance IFN-stimulated target gene expression, which could play a role in antiviral activity (14). Studies with HBV have shown that IL-1 affects HBV gene transcription in hepatocytes (19). However, the effect of IL-1 on HCV-infected hepatocytes has not been evaluated thoroughly.

IL-1 receptors are present on the membranes of human hepatocytes. They are capable of initiating the intracellular signaling cascade, which can potentially modulate the cellular physiological state (3, 30). We hypothesize that IL-1 may initiate a direct effect on viral RNA replication machinery in human hepatocytes. It has been difficult to ascertain the direct role of IL-1 in HCV replication due to the lack of an efficient cell culture system. The recent development of an HCV subgenomic viral RNA replicon provides a suitable model to test our hypothesis (2, 12, 21). The HCV replicon contains the HCV 5' nontranslated region (5'NTR) fused to 12 amino acids of the capsid coding region, the neomycin phosphotransferase gene (neo) directed by the HCV internal ribosome entry site (IRES), HCV nonstructural proteins NS3 to NS5B directed by an IRES from ependymomyocarditis virus, and the HCV 3'NTR. Using this model, it has been demonstrated that IFN-α has direct antiviral activity in hepatoma cells (12). In this study, using this replicon cell culture system, we examined the effects of IL-1β on HCV RNA replication and the associated intracellular signaling pathways.
IL-1 inhibits HCV subgenomic RNA replication. To investigate the direct antiviral activity of IL-1, we first incubated the replicon cell line FCA1 with different doses of IL-1β. The cells were harvested at different times for total RNA extraction. Total cellular RNA was extracted with TRIzol reagent, and 10 μg of RNA was used for Northern blot analysis. The 251-bp cDNA probe representing HCV 5′NTR generated by PCR was used to detect HCV subgenomic RNA. The 355-bp cDNA probe was used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as an internal control for total RNA loaded on the gel. We also examined the effect of IL-1 on viral protein production by Western blot analysis and immunohistochemistry using an NS5A-specific monoclonal antibody.

As shown in Fig. 1a, the replication level of the HCV subgenomic RNA replicon was significantly suppressed by treatment with IL-1β. The inhibitory effect was dose and time dependent, as shown in Fig. 1b. The inhibitory effect was more pronounced after 3 days of incubation. Consistent with the Northern blot result, HCV protein NS5A detected by an immunohistochemical stain using a monoclonal antibody against NS5A protein was also reduced by treatment with IL-1β (Fig. 1c).

To further confirm that expression of NS5A decreased in FCA1 cells treated with IL-1β, Western blot analysis was also performed. After FCA1 cells were treated with IL-1β for 2 days, total cellular protein was extracted and 50 μg of protein was used for electrophoresis. Western blotting was performed using chemiluminescent Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, Calif.) according to the manufacturer’s instructions. As shown in Fig. 1d, the levels of HCV NS5A protein expressed in FCA1 cells treated with IL-1β decreased significantly from the levels in the control FCA1 cells. The suppression level of 10 ng of IL-1β per ml is comparable to that of 10 ng of IFN-α per ml. The IL-1β concentration used in our experiments showed no apparent toxic effect to the cells. The overall cell numbers were similar for cells treated with IL-1β for 3 days and the corresponding controls, suggesting that the suppression of viral replication is the result of intracellular antiviral effect. The immunohistochemical staining in Fig. 1c clearly demonstrated the reduced viral protein expression in the cytoplasm. These results indicated that IL-1 directly inhibits HCV RNA replication machinery within human hepatoma cells.

IL-1 antiviral activity is associated with ERK MAP kinase activation. It is believed that binding of IL-1 with its receptor leads to activation of IL-1 receptor-associated kinases IRAK-1 and IRAK-2, followed by activation of mitogen-activated protein (MAP) kinase pathways, including extracellular regulatory kinase (ERK) and JNK pathways, and ultimately to the activation of NF-κB (1). To determine whether such signaling pathways are relevant in IL-1-induced antiviral activity in human hepatoma cells, we examined whether IL-1 antiviral activity is associated with the activation of ERK MAP kinase. We therefore analyzed the ERK protein phosphorylation status by Western blot analysis using a monoclonal antibody against phosphorylated ERK (p42/44) or an antibody against ERK. The parental Huh7 cells and the replicon FCA1 cells were treated with 10 ng of IL-1β per ml for 30 min, followed by protein extraction and Western blot analysis. As shown in Fig. 2, phosphorylated ERK was up-regulated at least three- to fourfold by IL-1β treatment. The basal levels of phosphorylated ERK in Huh7 cells and FCA1 cells are comparable, suggesting that either viral replication itself or viral nonstructural proteins have no significant effect on ERK activation. These results suggested that activated ERK may be responsible for the antiviral activity. To further determine the causal relationship, an inhibitor of the ERK MAP kinase cascade was added before the IL-1β treatment.

FIG. 1. Effects of IL-1 on HCV RNA replication and viral protein expression. (a) Northern blot analysis of total cellular RNAs from FCA1 cells treated with IL-1β. GAPDH served as an internal control. RNA from parental Huh7 cells served as a negative control. The positive control was subgenomic HCV replicon RNA generated by in vitro transcription. (b) Graphic representation of the Northern blot results. Cells were treated with IL-1β or IFN-α for 24 h and RNA extracted and loaded on a gel as described in (a). (c) Immunohistochemical staining of cells treated with IL-1β or IFN-α and stained with a monoclonal antibody against NS5A. The parental Huh7 cells are shown for a negative control. Bar, 20 μm. (d) Western blot analysis. Approximately 50 μg of total protein from FCA1 cells treated with IL-1β or IFN-α for 24 h was resolved on a gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a membrane. FCA1 cells were treated with 10 (lane 3) or 20 (lane 4) ng of IL-1β per ml or with 10 (lane 5) or 50 (lane 6) U of IFN-α per ml. Huh7 (lane 1) and FCA1 (lane 2) cells not treated with IL-1β or IFN-α were controls. An anti-HCV NS5A monoclonal antibody was used to detect NS5A protein. Actin served as an internal control.
used to specifically block the activation of ERK. The flavone compound 2-(2′-amino-3′-methoxyphenyl)oxanaphthalen-4-one (PD98059) specifically inhibits mammalian MEK-1/2 by binding to a regulatory site on the enzyme and has been extensively used in investigations of the function of the ERK MAP kinase pathway (1, 18). As shown in Fig. 2, PD98059 could effectively inhibit IL-1-associated ERK phosphorylation.

To determine whether inhibition of the ERK cascade would affect viral RNA replication and IL-1-induced antiviral activity, total RNA was extracted from cells treated with IL-1β in the presence of the PD98059 inhibitor and viral RNA replication levels were determined by Northern blotting. In addition, all cellular proteins were also extracted from these cells for Western blot analysis to determine viral NS5A protein expression. As demonstrated in Fig. 3, PD98059 inhibitor abolished IL-1-associated ERK phosphorylation.

As demonstrated in Fig. 3, PD98059 inhibitor abolished IL-1-induced suppression of viral RNA replication and viral NS5A protein expression, suggesting that IL-1 antiviral activity is dependent on activation of ERK. Moreover, PD98059 treatment alone, which down-regulated the basal level of phosphorylated ERK, also enhanced viral replication efficiency, suggesting that activated ERK has an inhibitory effect on viral replication. The MAP kinase pathway has been implicated as a mechanism by which signals are transduced from the cell surface to the nucleus in response to a variety of different stimuli. The MAP kinase pathway participates in intracellular processes by further inducing phosphorylation of intracellular substrates, such as other protein kinases and transcription factors. The activation of this pathway may also affect the phosphorylation status of HCV proteins (27). Whether IL-1 functions by regulating HCV protein phosphorylation remains to be investigated. Our experiment clearly demonstrated the causal relationship between the antiviral effect of IL-1 and activation of the ERK pathway.

**IL-1 induces expression of IFN-responsive gene 1-8U but not of 6-16 gene.** Our results demonstrated that IL-1β decreased HCV replication in the HCV replicon cell line through activation of the ERK MAP kinase pathway, suggesting that target genes downstream of ERK may be responsible for the antiviral effect. ERK activates several genes in cells (13). The target genes responsible for the antiviral effect are not known. At this point in time, antiviral proteins have been characterized only in IFN-induced systems (17, 28). It is generally believed that IFN antiviral activity is performed by several IFN-stimulated genes (ISGs). The well-characterized proteins include double-stranded RNA-activated protein kinase (PKR), Mx, and RNase L (31). Mx protein has been shown to have no effect on HCV RNA replication (10). However, the functions of most of the ISGs are still not known (7). We have shown that several genes are activated by IFN-α in Huh7 and FCA1 cells (H. Z. Zhu and C. Liu, submitted for publication). Of these genes, 6-16 and 1-8U genes have higher expression levels in FCA1 cells treated with IFN-α. These two genes may play a role in the establishment of the intracellular antiviral state.

To determine whether IL-1β affects the 6-16 and 1-8U genes, reverse transcription-PCR was performed using specific primers for each gene. As shown in Fig. 4a, IFN induced expression of both 6-16 and 1-8U genes, while IL-1β induced only 1-8U expression, not 6-16 expression. That 1-8U expression was induced by IL-1β was further confirmed by a Northern blot analysis. As shown in Fig. 4b, the level of 1-8U expressed after treatment with 20 ng of IL-1β per ml was at least 30-fold higher than that for the control. The results indicated that IL-1 can actually activate genes that are considered ISGs, suggesting that some overlap signaling pathways exist. The biological functions of both 6-16 and 1-8U are not known (26).
To determine whether 1-8U has antiviral activity, we cloned the 1-8U gene in the expression vector pEF6TOPO (Invitrogen, Carlsbad, Calif.) and transfected it into FCA1 cells. As shown in Fig. 5, 1-8U reduced HCV RNA by approximately twofold compared to the cells transfected with vector controls. This result suggested that 1-8U has antiviral activity. The mechanisms underlying the interactions of the 1-8U protein with viral replication machinery remain to be determined. It would also be interesting to see how the regulatory elements of the 1-8U gene respond to IL-1 and IFN stimulation. Interaction between IL-1 and IFN signaling pathways has been reported recently. One report suggested that IL-1 inhibited MX protein expression in mouse liver (29). Another report indicated that IL-1 potentiated IFN-induced 2’5’-oligoadenylate synthetase and PKR gene expression (14). Our results suggested that IL-1 alone could activate IFN-responsive gene 1-8U. All these observations support the notion that IL-1 and IFN may share some common pathways in initiating an antiviral state. Whether and how IL-1 can induce such intracellular pathways to inhibit HCV replication remain to be investigated.

In conclusion, our study provides the first evidence of direct antiviral IL-1 activity in human hepatoma cells. The activity seems to be associated with the activation of the ERK MAP kinase pathway. We demonstrated that the IFN-α-stimulated gene 1-8U was induced by IL-1β, while the ISG 6-16 was not, suggesting that IL-1 and IFN may have common and diverse antiviral pathways in hepatocytes. Moreover, we also showed that 1-8U protein had antiviral activity. Although IL-1 is a cytokine that causes tissue inflammation, its potent antiviral activity could be employed for antiviral treatment in patients with chronic HCV infections, particularly in the setting of IFN resistance. Further work should be aimed at enhancing IL-1 antiviral activity while limiting its proinflammatory activity.

We thank Christopher Seeger and Ju-Tao Guo for the cell line, Charles Rice for the HCV replicon plasmid, Johnson Lau for the anti-NS5A antibody, and James Crawford, Nao Terada, and David Nelson for helpful discussions.

C.L. was partially supported by a grant from the National Institutes of Health (K08 DK02958) and received The Charles Trey MD Memorial Liver Scholar Award from the American Liver Foundation.

REFERENCES


