Brief Report

Hepatitis C Virus Infection Downregulates the Ligands of the Activating Receptor NKG2D

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Natural killer (NK) cells are a major component of the host innate immune defense against various pathogens. Several viruses, including hepatitis C virus (HCV), have developed strategies to evade the NK-cell response. In our study, we found HCV infection could trigger DNA damage response by both ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) pathways. Recent reports had revealed that NKG2D ligands (NK cell-activating ligands) were upregulated when a major DNA damage checkpoint pathway was activated. However, here we found that DNA damage response was activated but NKG2D ligands were downregulated upon HCV infection. Further studies showed that the protease NS3/4A of HCV which had been shown relation with immune invasion contributed to the reduced expression of NKG2D ligands. These findings provide a novel insight into the mechanisms evolved by HCV to escape from the NK cell response. *Cellular & Molecular Immunology*. 2008;5(6): 475-478.

Key Words: NK cell, HCV, NKG2D ligand, DNA damage response, NS3/4A

Introduction

Natural killer (NK) cells are well recognized for their ability to provide the first line of defense against viral pathogens and they are increasingly being implicated in immune responses against microbe infections. NK cells are distinct from other innate immune cells and lymphocytes by their unique mode of target recognition. After binding to potential target cells, NK cell activating and inhibitory receptors interacted with ligands and transmitted signals, and then all the signals were integrated to determine whether NK cells stay or respond (1, 2). Inhibitory receptors include a variety of killer cell immunoglobulin-like receptors (KIRs) and C-type-lectin receptors such as CD94/NKG2A，and activating receptors include NKG2D (3). NKG2D receptor expressed by NK cells and activated $CD8^+$ T cells recognized self-molecules (NKG2D ligands) that are upregulated in diseased cells by poorly understood mechanisms. Raulet reported mouse and human NKG2D ligands were upregulated when a major DNA damage checkpoint pathway initiated by ATM (Ataxia telangiectasia mutated) or ATR (ATM- and Rad3-related) was activated.

The induction of cell cycle checkpoints and activation of the ATM/ATR dependent pathway have been reported to accompany infection by a number of different viruses. Recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation (4). ATM-dependent DNA damage response was activated in the presence of HCV genome (5), while the statue of DNA damage response by HCV virus infection and NKG2D ligand expression were still unknown. Here we showed that HCV infections led to activated ATR-ATM DNA damage response, but NKG2D ligands were downregulated, further studies suggested that NS3/4A contributed to the reduced levels of NKG2D ligands.

Materials and Methods

Cell culture, infection and HCV RNA detection

The human hepatocyte cell line HL7702 was obtained from Shanghai Biochemistry Institute. Serum samples from HCV carriers were analyzed. The patients infected with HCV were diagnosed by ELISA (SIIC Ke-Hua, Shanghai), and HCV-RNA in the serum sample was quantified using FQ-PCR (Corp). Normal serum was obtained from healthy non-infected individuals as control. HCV infection was

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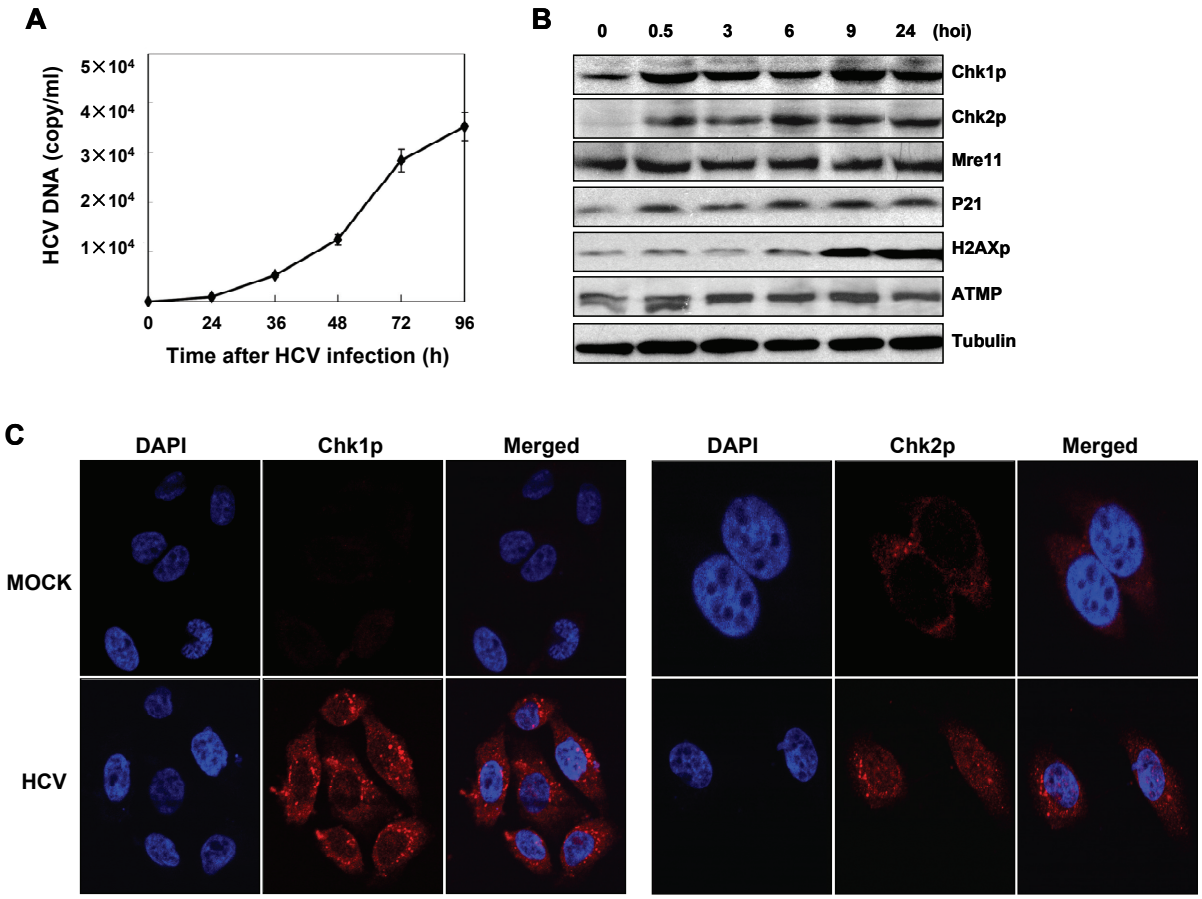

Figure 1. Activated ATM/ATR dependent DNA damage responses in HCV infected cells. (A) HL7702 cells (10^5) in a 6-cm plate were infected with 10^6 virus particles from HCV-positive serum at 37°C under 5% CO_2. Normal serum from healthy individuals was used as non-infected control. Prior to cell harvesting, the cells were washed 8 times thoroughly to remove excess of the viral input. RNA was extracted at various times of infection (hours of infection, hoi) and subjected to FQ-PCR assay. (B) The experiments were carried out as described in (A), except that whole cell lysates were prepared at various times of infection (hours of infection, hoi) and subjected to an immunoblotting assay by using antibodies to the indicated proteins. Tubulin was used as the equal loading control. (C) HL7702 cells were infected with HCV-positive serum for 0.5 h. Normal serum from healthy individuals was used as non-infected control. Immunofluorescences with antibodies to Chk1 ser-345, Chk2 ser 68 (red) were monitored. DNA was stained with DAPI (blue).

monitored by culturing 10^5 HL7702 cells in a 6-cm plate in 3 ml RPMI-1640 containing 10^6 HCV virus particles. The cells were washed thoroughly 8 times to remove excess of viral inputs before harvesting. HCV RNA level was quantitated by the FQ-PCR method as described previously (6).

Immunoblotting assay

Whole cell lysates were extracted and resolved on a 7-15% SDS-PAGE gel as usual. Tubulin from Sigma, P21, ATM phosphoserine 1981 (ATMp), Chk2 phosphothreonine 68 (Chk2p), Chk1 phosphoserine 345 (Chk1p), and H2AX phosphoserine 139 (H2AXp) from Cell Signaling Technology were used according to the manufacturer's protocals.

Immunofluorescence assay

HL7702 cells were grown on glass coverslips for 18 h prior to infection. At 24 h post infection, cells were immunostained with anti-Chk1p and Chk2p and the nuclei was stainined with DAPI.

Flow cytometry

Cells were harvested by trypsinization, and fixed with 4% paraformaldehyde. Anti-MICA, MICB and ULBP1 (R&D system) antibodies were diluted to 25 μg/ml and 10 μl of the diluted solution was add to 1-2.5 × 10^5 cells in a total reaction volume of 100 μl. The binding of unlabeled monoclonal antibodies was visualized by adding FITC labeled goat anti-mouse IgG. Thereafter, cells were washed twice and analyzed by FACSAria (Becton Dickinson).

Results

HCV infection induced a cellular DNA damage response dependent on both ATM and ATR

Although it has been proposed the HuH-7-derived cells (7, 8)

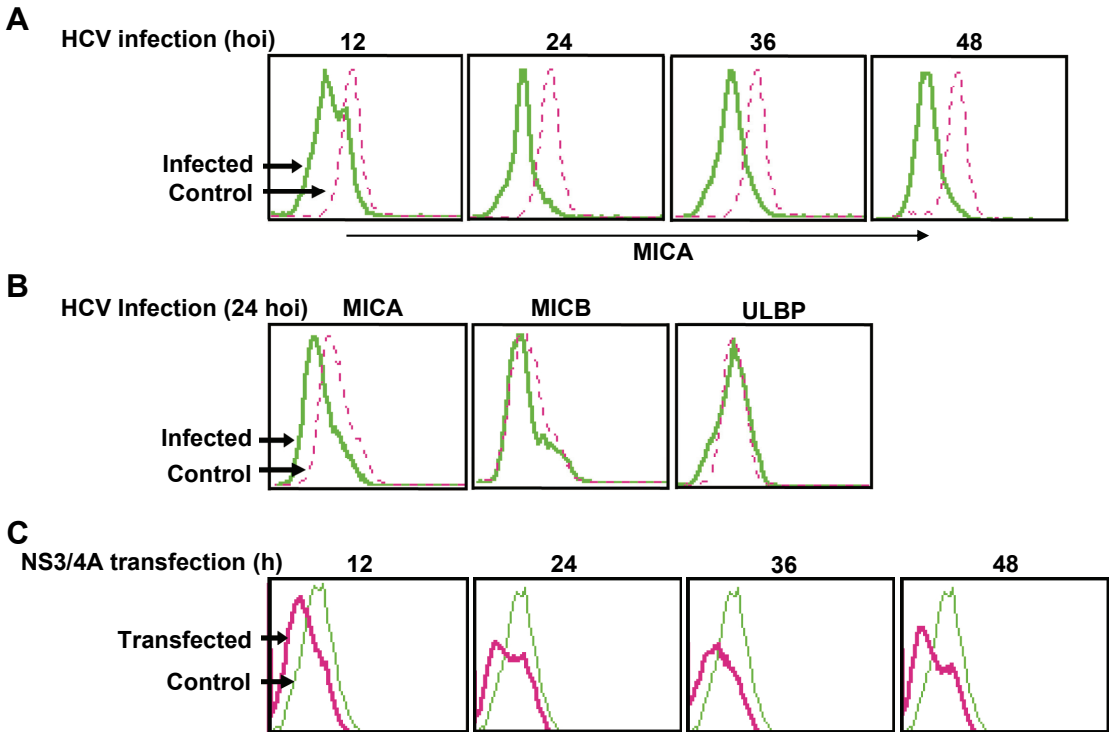

Figure 2. Downregulation of cell-surface NKG2DLs in HCV infected cells. (A) HL7702 cells were incubated with HCV-positive serum. Normal serum from healthy individuals was used as non-infected control. FACS with antibody to MICA was monitored at different times after HCV serum addition. (B) HL7702 cells were incubated with HCV-positive serum. Normal serum from healthy individuals was used as non-infected control. FACS with antibodies to MICB and ULBP2 was monitored at different times after HCV serum addition. (C) HL7702 cells were transfected with plasmids encoding NS3/4A. Twenty-four hours after transfection, cells were fixed and examined by flow cytometry with antibodies to MICA. Empty vector was used as non-infected control.

harboring a replicative genome-length HCV RNA activate ATM-dependent damage response pathway, little is known about whether natural HCV virus infection could also activate ATR or ATM dependent damage response pathway. We incubated HL7702 cells with HCV-positive serum and total RNA was extracted to test whether HCV could propagate in HL7702 cells. FQ-PCR showed that HCV DNA increased with time after HCV virus infection, suggesting that HL7702 supports the infection and replication of HCV (Figure 1A). To explore whether acute cellular DNA damage response was induced upon HCV infection, whole cell lysates from the HCV-infected and non-infected cultures were examined for the phosphorylation status of the DNA damage response proteins. We observed significant Chk2 phosphorylation at threonine 68 and Chk1 phosphorylation serine at 345, two specific markers for ATM and ATR activation respectively (Figure 1B). We then used immunofluorescence to examine the localization of these proteins after infection. As shown in Figure 2B, Chk1 phosphorylation foci as well as the Chk2 phosphorylation foci in non-infected cells were very faint, as opposed to the HCV infected cells where the foci were larger, more numerous and much brighter. These results showed that HCV infection triggered ATM and ATR dependent DNA damage response pathway.

HCV infection downregulated cell-surface expression of NKG2D ligand

The DNA damage response, which has previously been shown to arrest the cell cycle, enhance DNA repair functions or trigger apoptosis, may also participate in alerting the immune system to the presence potentially dangerous cells. Mouse and human NKG2D ligands are upregulated in conditions known to activate a major DNA damage checkpoint pathway initiated by ATM or ATR DNA damage response. Therefore, we tested the alteration of NKG2D ligands upon HCV infection. Surprisingly, instead of increased NKG2D ligand expression, the cell surface expression of NKG2D ligand MICA was sharply reduced. As shown in Figure 2A, MICA was downregulated beginning at 12 h and decreased greatly at 48 h after infection. MICB and ULBP1 are another two members of NKG2D ligands, while their expressions were almost unchanged (Figure 2B).

HCV protease NS3/4A downregulated cell-surface expression of NKG2D ligand

Serine protease NS3/4A expressed by HCV blocks virus induced activation of IFN regulatory factor 3 (IRF-3), TRIF (9) and MAVS (10). NS3/4A, therefore, limits expression of multiple host defense genes and is related to immune evasion. So we proposed that NS3/4A might contribute to reduced

expression of NKG2D ligand by HCV infection. As shown in Figure 2C, NS3/4A transfection led to reduced cell-surface expression of MICA.

Discussion

Many works have suggested that the cellular DNA repair machinery can recognize viral genetic material as damage (11). The induction of cell cycle checkpoints and activation of the ATM/ATR dependent pathway have been reported to accompany infection by a number of different viruses. A very recent report showed that HCV genome transfected cells maybe trigger ATM-dependent DNA damage response (5). This infection model system does not allow the study of viral entry and the earliest events in the HCV life cycle (12). In our study, HL7702 cells were infected with HCV-positive serum mimicking the natural HCV infection process. We propose that HCV infection induces acute cellular DNA damage response dependent on both ATM and ATR, as evidenced by increased phosphorylation of Chk1 and Chk2.

It has been demonstrated that the DNA damage response alerts the immune system by inducing expression of cell surface ligands for the activating immune receptor NKG2D, which is expressed by NK cells and some T cells. HCV trigged the DNA damage response, while the expression of NKG2D ligand was downregulated. Recent reports have suggested that viruses have evolved numerous strategies to evade the activation of NK cells and have influenced the evolution of NK cell receptors and their ligands. Some virus, for example, HIV (13), HBV (14) and CMV (15) have developed strategies to evade the human NKG2D receptor and some of them have evolved strategies to interfere the expression of the human NKG2D ligands. With respect to NK activity in HCV infection, *in vitro* experiments have revealed that HCV glycoprotein E2 is capable of binding to CD81 and thus inhibiting NK cell activity (16, 17). Here we provide another mechanism that contributes to HCV invasion of NK cells. NS3/4A is one of the important genes that play key roles in immune evasion; our studies provide further evidence that NS3/4A expression lead to the downregulation of NKG2D ligand. However further investigation should be done to explain this phenomenon.

In this study, it was shown for the first time that HCV infection downregulated NKG2D ligands MICA and MICB. Further study showed this downregulation was due to the expression of serine protease NS3/4A of HCV. Such findings were obtained in HCV infected cells which is more similar to HCV infection *in vivo*. As NK cells are specialized lymphocytes that provide the first line of defense through killing pathogen-infected cells and transformed cells, our study might shed light on the underlying mechanism of HCV persistent infection.

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