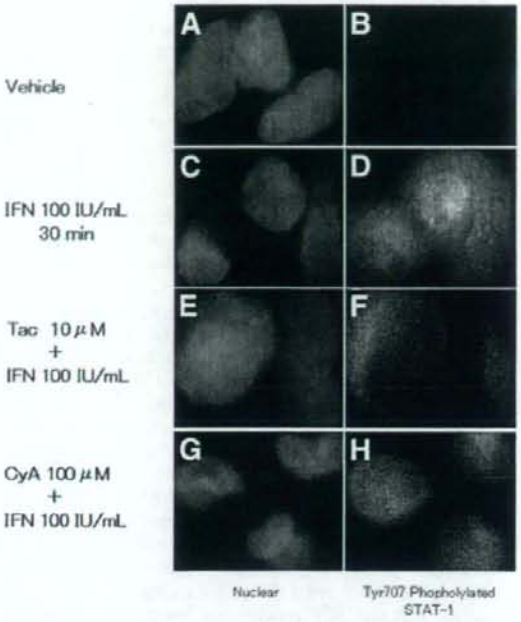

Figure 6. Inhibition of IFN-α-induced nuclear translocation of phosphorylated STAT-1 by calcineurin inhibitor. Hc cells were pretreated in the absence (A-D) or presence of 10 μmol/L Tac (E,F) or 100 μmol/L CyA (G,H). After pretreatment, Hc cells were stimulated by 100 IU/L IFN-α (C-H) for 30 minutes. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (B,D,F,H) and Hoechst staining (A,C,E,G), and visualized with fluorescence microscopy. The results shown are from one representative experiment from a total of three performed.

The total IFN-α-stimulated tyrosine phosphorylated STAT-1 was decreased by pretreatment with Tac; furthermore, the nuclear translocation rate of activated STAT-1 was inhibited both by pretreatment with Tac and CyA. However, in the case of pretreatment with Tac and CyA, there was no effect on the nuclear translocation of tyrosine phosphorylated STAT-2. Secondarily, we evaluated the location of tyrosine phosphorylated STAT-1 by fluorescence immunohistochemistry of cultured Hc cells (Fig. 6).The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was observed, but its translocation was inhibited by pretreatment with Tac. Along with the nuclear translocation rate of activated STAT-1 by western blotting (Fig. 5), pretreatment with Tac also attenuated the nuclear staining of activated STAT-1 compared to IFN-α alone, but did not attenuate the expression of activated STAT-1 by immunohistochemistry.

Inhibitory Effect of Tac on IFN-α-Induced Anti-HCV Efficiency

To examine the effect of calcineurin inhibitors on IFN-α, we used the full-length HCV replication system, OR6 cells. The cells were treated with IFN-α after 16 hours in

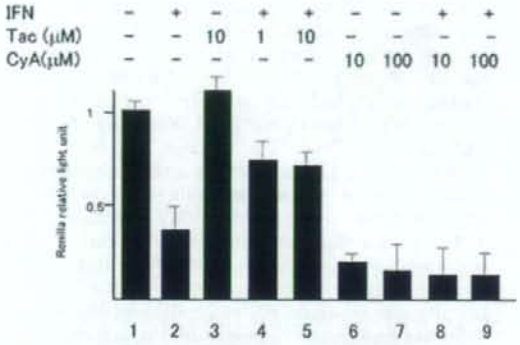

Figure 7. Alternation of IFN-α-suppressed HCV replication by Tac. OR6 cells, full-length replicon system, were treated with 100 IU/mL of IFN-α in the absence (lane 2) or presence of pretreatment (lanes 4, 5, 8, and 9). Indicated concentration of calcineurin inhibitor alone was lanes 3, 6, and 7, lane 1 was not treated with IFN-α and calcineurin inhibitors. One day later, *Rennila* luciferase activity was determined by luminometer.

the presence or absence of pretreated Tac or CyA (Fig. 7). IFN-α or CyA alone repressed the *Renilla* luciferase activity, which is well correlated with HCV-RNA concentration in OR6 cells.[19] In contrast, Tac alone had little effect on *Renilla* luciferase activity. However, pretreatment with Tac attenuated the IFN-α-induced repression of *Renilla* luciferase activity (Fig. 7; lane 2 versus lanes 4 and 5), but pretreatment with CyA did not (Fig. 7; lanes 8 and 9).

DISCUSSION

We herein show that calcineurin inhibitors, especially Tac, are negative regulators of IFN signaling in the hepatocyte, and the greatest cause of this phenomenon is phosphorylation of STAT-1, next to inhibition of nuclear translocation of STAT-1. Disturbance of STAT-1 phosphorylation caused diminished ISRE-containing promoter activity, for example PKR and STAT-1, and antiviral protein expression declined. Pretreatment with Tac diminished the replication inhibitory effect of IFN-α. This phenomenon has a detrimental effect on IFN therapy after HCV-related liver transplantation. In our experiments, we speculated that Tac is not better suited for posttransplantation IFN therapy than CyA, but it did not report that IFN-a response is different between Tac and CyA in human study in previous time. When the alternative of potent immunosuppressant for prevention of rejection, or antiviral-activity for HCV reactivation is weighed, we might need to consider other factors in choosing between Tac and CyA. We had compared high concentration CyA with low concentration Tac, since rejection was controlled by serum trough values of tacrolimus of 5 ng/mL and of cyclosporin of 100 ng/mL in our hospital in the period of stability after liver transplantation.

Recently, the difference between Tac and CyA has been regarded in another function than immunosuppression, and we presume that this discrepancy de-

pended on differences of "immunophilins." Immunophilins are a ubiquitous family of proteins. All cells contain several members of this family, which bind specific calcineurin inhibitors and participate in many cellular functions.[22] Tac has been reported to have neuroprotection,[23] but CyA did not, whereas CyA had anti-HCV action,[24-26] but Tac did not. Tac binds specific FK506 binding protein members of the immunophilin family, whereas cyclosporin binds a different subset of immunophilins (cyclophilins). FK506 binding protein and CyP have the same function as peptidyl prolyl *cis-trans* isomerase and they inhibited the nuclear translocation of nuclear factor of activated T cells (NF-AT). Despite this common pathway, the cell protection activity has been reported to require the induction of heat shock protein 70 by Tac but not CyA,[27] and the anti-HCV activity contributed to a specific blockade of CyP B by CyA.[25] The differences in the medical effects for immunosuppression between Tac and CyA require attention, when these immunosuppressants are used in posttransplantation-related HCV infection.

In our study, the IFN-induced tyrosine phosphorylated STAT-1 and -2 both decreased after the administration of Tac, but Tac is known essentially for the inhibition of serine/threonine protein phosphatase. Calcineurin, regardless of independent Jak-1 tyrosine phosphorylation, and CyA did not have such a tyrosine phosphatase action against STAT-1 and -2. We could not resolve this phosphatase mechanism, but we speculated that Tac induced the tyrosine phosphatase kinase and inhibited tyrosine phosphorylation of STAT-1 and -2. Tac did not induce suppression of cytokines signaling-1 and 3, Jak inhibitors, by western blotting in our study (data not shown); however, we could not rule out the induction of other types of tyrosine phosphatase. Previous studies described that suppressor of cytokines signaling-1, 3 and SH2-containing protein tyrosine phosphatase inhibited NF-AT activation,[28-30] and therefore the relationship between Tac and tyrosine phosphatase might be reconsidered. Barat and Tremblay[31] and Zhu and McKeon[32] previously described the protein-tyrosine phosphatase inhibitor bisperoxovanadium as a potent activator of T cell receptor signaling, and SH2-containing protein tyrosine phosphatase-1, T cell protein-tyrosine phosphatase, Tac, and CyA are inhibitors of such activation. We were interested in the inhibition of protein-tyrosine phosphatase inhibitor by Tac and CyA, because Tac and CyA possessed the same action as SH2-containing protein tyrosine phosphatase-1 and protein-tyrosine phosphatase.[32] Furthermore, this action of Tac was stronger than CyA.[31] From these studies, we assume that Tac has tyrosine phosphatase action in the hepatocyte and inhibits tyrosine phosphorylation of STAT-1 and -2.

The inhibition of IFN-induced antiviral proteins by Tac, and the inhibition of nuclear trafficking of tyrosine phosphorylated STAT-1, is the common phenomenon between Tac and CyA in this study. This phenomenon was observed in the western blotting findings (Fig. 3) and immunohistochemistry of the cultured cells (Fig. 6).

NF-AT activation requires the suppression of Crm1-dependent export from nucleus to cytoplasm by calcineurin,[33] and the presence of importin, bounded to calcineurin, in the nucleus.[34] In IFN-induced Jak-STAT signaling, nuclear trafficking of ISGF-3 requires suppression of Crm1 and binding importin[18] in the same fashion as NF-AT. Calcineurin inhibitors bind to immunophilin and inhibit dephosphorylation of NF-AT, then they inhibit the transcription activity of NF-AT. In addition to such action, it might be considered that the nuclear trafficking of NF-AT is regulated by the calcineurin inhibitor and immunophilin complex. We speculated that the decrease of the nuclear import of tyrosine phosphorylated STAT-1 is the function, the calcineurin inhibitor and immunophilin complex modified Crm1 and importin in the same fashion as NF-AT. Then, we recognized that the mechanisms of diminished tyrosine phosphorylation STATs and nuclear translocation STAT-1 were different.

Presently, there is no definite opinion regarding the selection of calcineurin inhibitors for liver transplantation.[6] However, reports of inhibition of HCV replication by CyA in vitro were noted recently[24-26] and the result were same in our full-length replicon system (Fig. 7). In our data, we consider that CyA has the effect of, not only the previously reported anti-HCV replication action itself, but it creates much less interference with IFN treatment for HCV reactivated after liver transplantation than does Tac. It has been reported that CyA increased the chance of a sustained viral response after liver transplantation.[35] However, we used care with our data, because both Tac and CyA inhibit the nuclear translocation of tyrosine phosphorylated STAT-1. Our data revealed that when an excess of CyA was used after liver transplantation, it resulted in a decrease in the amount of IFN-induced antiviral protein, because of inhibition of nuclear transportation of tyrosine phosphorylation STAT-1 (Figs. 5 and 6). The immunosuppression levels of Tac and CyA have already been reported to decrease significantly in patients responding favorably to anti-HCV therapy post–liver transplantation.[36] In this study, we therefore considered it necessary to pay attention to an excess dose of CyA, when IFN treatment for reactivation of HCV is required.

In conclusion, Tac has been shown to influence the tyrosine phosphorylation of STAT-1, and the result was a decline in antiviral protein PKR. In addition, Tac and CyA have been shown to interfere with the translocation of STAT-1. We speculated that posttransplantation immunosuppression is part of the reason for IFN resistance to HCV reinfection of the graft liver. As the course, calcineurin inhibitors, especially Tac, were pointed out in this study, and we clarified a part of the IFN resistance. Although the mechanism of inhibition of IFN signaling has not yet been fully investigated, it is necessary to compare the antirejection action of Tac to the anti-HCV action of CyA when selecting calcineurin inhibitors.

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Possible Molecular Mechanism of the Relationship Between NS5B Polymorphisms and Early Clearance of Hepatitis C Virus During Interferon Plus Ribavirin Treatment

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We previously reported the relationship between viral polymerase polymorphisms and the initial decline in viral load induced by interferon-α and ribavirin therapy in genotype 1b-related chronic hepatitis C patients. The presence of E124K and I85V of NS5B was closely associated with viral clearance at 8 weeks of treatment. The aim of this study was to investigate the mechanisms by which this polymorphism of NS5B protein affects early viral clearance. We used a replicon system derived from strain O, genotype 1b virus. Three mutants (V85I), (K124E), and (V85I/K124E) were introduced to the replicon. OR6c, a derivative of HuH7 cells, was transfected with the replicon including a luciferase reporter gene. Luciferase activities were measured 72 hr post-transfection. All three mutants showed higher luciferase activity than that of the wild type, and the V85I mutant showed the highest activity. This result was also confirmed by neomycin gene-containing replicons with same mutations. All replicons were down-regulated by ribavirin, but the level of reduction in the V85I mutant was the lowest. Our results suggested that this mutation at least partly contributes to resistance to early viral clearance during interferon and ribavirin combination therapy. ***J. Med. Virol. 80:632–639, 2008.***

KEY WORDS: hepatitis C virus; NS5B polymorphism; replicon; interferon and ribavirin combination therapy; viral proliferation

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health. An estimated 65–80% of the individuals infected with HCV develop persistent infection while 20–50% develop cirrhosis and 5% develop hepatocellular carcinoma (HCC) [Liang et al., 2000; Gao et al., 2004]. Until recently, interferon (IFN)-α and IFN-β were the only available treatments for HCV infection, although only 10–15% of treated subjects achieved sustained viral eradication with IFN monotherapy, and early viral clearance after initiation of IFN monotherapy was correlated with sustained viral clearance [Saito et al., 2000].

The current approved treatment for HCV infection is pegylated IFN-α (peg-IFN) in combination with ribavirin (RBV). This combination therapy leads to viral clearance in 50–80% of cases, depending on the infecting HCV genotype, and 50% of patients with HCV genotype 1b and high baseline levels of viral RNA do not achieve a sustained virological response with the combination therapy after 48 weeks [Manns et al., 2001; Fried et al., 2002; Feld and Hoofnagle, 2005]. Several prior studies have attempted to predict the efficacy of IFN plus RBV combination therapy. A quantitative measurement of HCV viremia or the initial decline in viral load is a reliable marker for early prediction of the therapeutic response to IFN and RBV combination therapy [Zeuzem et al., 1998; Bouvier-Alias et al., 2002; Takahashi et al., 2005; Lukasiewicz et al., 2007].

RBV is a broad-spectrum nucleoside analogue antiviral drug which is especially noted for its actions

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against RNA viruses and exhibits in vitro activity against some DNA and RNA viruses, including certain members of *Flaviviridae* [Sidwell et al., 1972]. It has recently been demonstrated that the antiviral activity of RBV can result from the ability of a viral RNA-dependent RNA polymerase (RdRP) to utilize RBV triphosphate and to incorporate this nucleotide into the viral genome with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus [Crotty et al., 2000; Lanford et al., 2003]. Moreover, RBV exhibits an antiviral effect through a mechanism of error-prone replication in the HCV subgenomic replication system [Contreras et al., 2002]. Although RBV by itself cannot decrease serum HCV RNA levels in patients, it has been demonstrated that combination therapy with RBV and either IFN-α or peg-IFN yields a higher sustained response rate than is achieved with IFN-α monotherapy [Pol et al., 2000; Poynard et al., 2000; Saracco et al., 2001].

We previously reported the relationship between viral RdRP polymorphisms and the initial decline in viral load induced by IFN-α and RBV therapy in genotype 1b-related chronic hepatitis C patients [Kumagai et al., 2004]. Substitution of glutamic acid to lysine at the 124th position (E124K) and of isoleucine to valine at the 85th position (I85V) of NS5B was closely associated with viral clearance at 8 weeks of treatment.

In this study, we used the genotype 1b HCV replicon system [Ikeda et al., 2005] to generate NS5B mutants (E124K, I85V, and both) and we compared the replication activity with that of the wild-type replicon and to analyze how this polymorphism of NS5B protein affects early viral clearance during combination therapy with IFN and RBV. We also examined the significance of NS5B polymorphisms in the RBV-induced decrease in viral replication. We concluded that the identified polymorphism of NS5B partly affects viral replication.

MATERIALS AND METHODS

Cell Culture System

OR6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM), in addition to G418 (300 μg/ml; Geneticin, Invitrogen), and were then passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described [Ikeda et al., 2005].

Plasmids

The plasmids pON/C-5B/KE (Fig. 1a) and pHCV-O were described previously [Ikeda et al., 2005]. This plasmid includes the adaptive mutation of K1609E of NS3 to enhance the efficiency of replication, this adaptive mutation was reported by Lohman et al. (22) The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. To introduce a pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/KE/(V85I&K124E), we first made PCR fragments including the partial NS5B region with the primers 5′-ggatcccgatctcagcgacgg-3′ and 5′-tctagaggtcccattcgccattac-3′. This 2.4-kb fragment was subcloned into pSTBlue1 Blunt vector (Novagen, Madison, WI) to generate pSTBlue-1MN002. Each vector expressing the V85I mutant, K124E mutant, and V85I&K124E double mutant of HCV-O was generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) to generate pSTBlueMN002(V85I), pSTBlueMN002(K124E) pSTBlueMN002(V85I&K124E). Next, pON/C-5B was

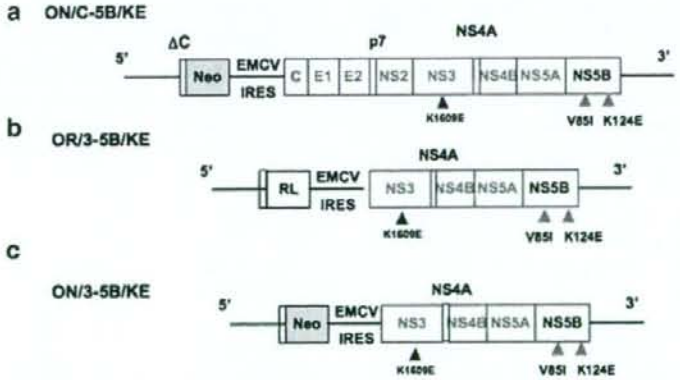

Fig. 1. **a**: Organization of genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES. This construct also contains adaptive mutation K1609E which is indicated by a black triangle. We use this construct as a wild type. Grey triangle is the position of 85 and 124 in NS5B which we generated mutation to the replicon for this experiments. **b**: The construct of the reporter subgenomic HCV replicon carries the renilla luciferase gene (RL). **c**: The construct of the reporter subgenomic HCV replicon carries the Neo gene.

digested with *Sna*b1 and *Xba*1 and subcloned into pSTBlue-1 to create pSTBlueMN001. All of the pSTBlue-MN002mutants were digested with *Bam*H1 and *Xba*1, which were subcloned into pSTBlueMN001 to create pSTBlueMN001mutant. The pSTBlueMN001 mutants were digested with *Sna*b1 and *Xba*1 and re-ligated in pON/C-5B/KE to introduce pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/(V85I&K124E). The plasmids pOR/3-5B/KE/(V85I), pOR/3-5B/KE/(K124E) and pOR/3-5B/KE/(V85I&K124E), were constructed from pOR/3-5B/KE (Fig. 1b) by swapping for fragments of pSTBlueMN001 mutants digested with *Sna*b1 and *Xba*1. DNA sequencing of the manipulated regions of the plasmids verified all mutations.

RNA Transfection and Selection of G418-Resistant Cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at 10^7 cells/ml in PBS. Twenty microgram of ON/C-5B/KE or its mutant derived RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 μg/ml G418. About 3 weeks after transfection and G418 selection, cells were fixed and stained with Coomassie brilliant blue (0.6 g/l in 50% methanol–10% acetic acid) and the number of colonies was counted.

Transient-Replication Assays With Luciferase Replicons

OR6c cells were transfected by electroporation as the same protocol described above using 20 μg of OR/3-5B/KE or its mutants derived RNAs carrying the renilla luciferase (RL) gene. After addition of 2 ml of complete DMEM, 2×10^4 of aliquot OR6c cells were plated in 24-well plates at least in triplicate for each assay and harvested at various time points with renilla lysis reagent (Promega KK, Tokyo, Japan) and subjected to the RL assay according to the manufacturer's protocol (Promega). Values obtained with cells harvested 6 hr after electroporation were used to correct for the transfection efficiency.

IFN and Ribavirin Treatment

To monitor the anti-HCV effect of IFN and RBV on replication, OR6c cells were transfected by electroporation using 10 μg of OR/3-5B/KE derived RNAs as described elsewhere [Crotty et al., 2000]. OR6c cells (2×10^4/well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 hr. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, and 20 units/ml or RBV at a final concentration of 50,100, and 200 μM for 72 hr, harvested with renilla lysis reagent (Promega), and assayed for luciferase activity according to the manufacturer's protocol. We also studied about the additional effect of RBV (100 μM) on IFN (1 u/ml).

Cell Viability

We checked toxic effect of IFN and RBV. Effect of IFN (1 and 4 units/ml), and RBV (50 and 100 μM) on cell viability was investigated. To examine the cytotoxic effect of IFN and RBV on OR6c cells with OR/3-5B/KE replicon RNA, the cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24-hr culture, the cells were treated with IFN or RBV at final concentrations of 2 and 4 units/ml or 50 and 100 μM, respectively, in the absence of G418. After incubation for 72 hr, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

Indirect Immunofluorescence

Cells were grown on four-well chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol–acetone (1:1, v/v) for 10 min at room temperature. Dilutions of primary murine monoclonal antibody to residues 21–40 of the core protein (2Zcp11; Tokushu Men-eki Institute, Tokyo) (1:1,000), were prepared in PBS containing 3% bovine serum albumin and incubated with fixed cells for 2 hr at room temperature. After additional washes with PBS, specific antibody binding was detected with a goat anti-mouse immunoglobulin G-fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Cells were washed with PBS, and mounted in DAKO Fluorescent mounting medium (DAKO Japan, Tokyo, Japan) prior to examination using a Zeiss AxioPlan2 fluorescence microscope.

Statistical Analysis

Difference in relative luciferase activity among mutant replicons and differences in anti-HCV activity of RBV among mutant replicons were tested using Student's *t*-test and Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered statistically significant.

RESULTS

Mutation in NS5B Enhances Levels of Replication on Transient Assay

To investigate whether the mutations in NS5B of the HCV genome affect replication, we used subgenomic HCV replicons with the renilla luciferase gene for transient assay [Ikeda et al., 2005]. In a previous study [Kumagai et al., 2004], substitution of glutamic acid at the 124th position with lysine and substitution of isoleucine at the 85th position with valine in NS5B yielded a complete match with the population of good

responders (5 out of 5 patients). We introduced mutations to two different types of replicon to obtain ON/C-5B/KE(V85I), ON/C-5B/KE/(K124E), ON/C-5B/KE (V85I&K124E), OR/3-5B/KE/(V85I), OR/3-5B/KE(K124E), and OR/3-5B/KE(V85I&K124E) as described in Materials and Methods Section. The subgenomic replicons with V85I showed higher replication activity than the wild-type replicon in OR6c cells (Fig. 2). Also the replicon with K124E and the replicon with V85I&K124E showed slightly higher replication activity than the wild type, but the replicon with K124E single amino acid mutation did not show statistically higher replication activity than the wild type (Fig. 2). We initially expected that double mutations (V85I&K124E) would lead to better replication than either of the single mutations (V85I or K124E), but interestingly the V85I mutation on NS5B replicated best. This result indicated that the level of replication was affected by amino acid substitution at the 85th position.

Mutation in NS5B Enhances the Efficiency of Colony Formation in Cured Cells

In colony formation assay, we used cured subgenomic replicon cells (OR6c), since cured cells enhanced colony formation of the replicon more efficiently than did parental HuH-7 cells. We examined the effect of these mutations in full-length replicon, ON/C-5B/KE by a colony formation assay. In the initial experiment, we introduced each 20 μg of RNA derived from the ON/C-5B/KE, ON/C-5B/KE/(V85I), ON/C-5B/KE/(K124E), and ON/C-5B/KE/(V85I&K124E) into OR6c cells. After 3 weeks of G418 selection at a concentration of 300 μg/ml, only one colony was obtained and the same result was obtained with ON/C-5B/KE(K124E) and ON/C-5B/KE/(V85I&K124E) transcripts. In repeated experiments, the number of G418-resistant colonies was reproducibly one or zero, but when ON/C-5B/KE/(V85I) transcripts was electroporated, G418 resistant 4–6 colonies was obtained in repeated experiments. These results also confirm that the replication level of ON/C-5B//KE/(V85I) is higher than that of ON/C-5B/KE, ON/C-5B/KE/(K124E) and ON/C-5B/KE/(V85I&K124E).

As the efficiency of colony formation with full-length replicon (ON/C-5B/KE; Fig. 1a) was quite low, we investigated colony formation with subgenomic replicon, ON/3-5B/KE (Fig. 1c). Figure 3 shows the representative result of three independent colony formation assays. The efficiency of colony formation of ON/3-5B/KE was better than that of full-length replicon and the colony formation of in vitro transcript of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 39 (61), 157 (132), 44 (54), and 134 (107), respectively (the numbers in parentheses show another set of result). The efficiency of colony formation of ON/3-5B//KE/(V85I) was greater than that of ON/C-5B/KE and it showed a similar result with that obtained from genome-length replicon.

Inhibition of HCV RNA Replication by IFN and RBV

We examined the inhibitory effect of IFN and RBV on the replication of OR/3-5B/KE. In this experiment, the subgenomic replicon system was used. OR6c cells were treated with IFN at concentrations of 1–20 μM (Fig. 4) and RBV at concentrations of 50, 100, and 200 μM (Fig. 5) after transfection of OR/3-5B/KE derived RNA. Since it is important to know how IFN and RBV treatment is toxic to the cells, we examined cell viability after treatment with 50 and 100 μM of RBV or 2 and 4 units/ml of IFN. The cell viability of OR6c was not

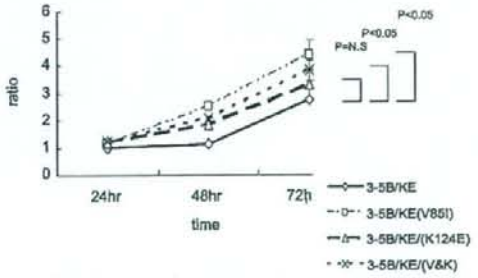

Fig. 2. Effect of amino acid substitutions in NS5B on transient replication activity of replicon. The replication activity of reporter subgenomic HCV replicon with mutation of V85I, K124E, or V85I and K124E (V&K) was compared with that of wild-type in OR6c cells (transient transfection). After 72 hr of transfection, the Renilla luciferase (RL) assay was performed as described in the Materials and Methods section. The relative RL activity (ratio) of mutants was calculated in comparison to that of subgenomic replicon of wild-type (assigned as 1). The data indicate means ± SD of triplicates from three independent experiments. 3-5B/KE: OR/3-5B/KE, 3-5B/KE(V85I): OR/3-5B(K124E), 3-5B/KE/(K124E): OR3-5B/KE/(K124E), 3-5B/KE/(V&K): OR/3-5B/KE/(V85I&K124E).

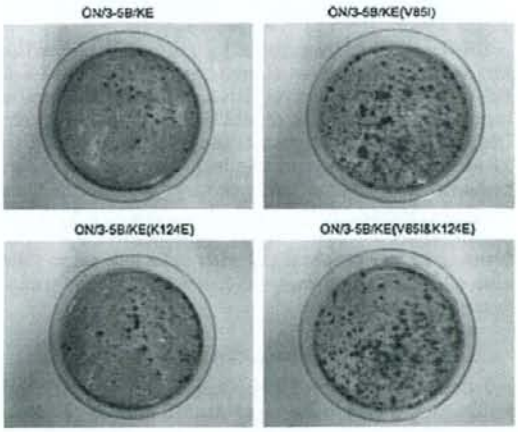

Fig. 3. Colony formation assay of OR6c cells transfected with wild-type and three different mutant replicons. A representative result of colony formation assay using subgenomic replicon RNA (ON/3-5B/KE) system. The efficacy of colony formation was much higher than that of full-length replicon RNA (ON/C-5B/KE). In this series of photographs, colony forming unit of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 2.7/μg, 6.7/μg, 3.0/μg and 5.4/μg, respectively.

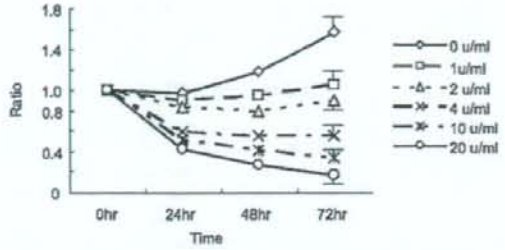

Fig. 4. Dose-dependent inhibition of replication by interferon-α (IFN). OR6 cells were transfected with wild-type replicon (OR/3-5B/KE). Inhibition of HCV RNA replication in the OR6c cell treated with IFN-α was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The cells were treated with IFN-α (0, 1, 2, 4, 10, and 20 u/ml), and the Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

changed by these treatments (Fig. 6), indicating that both IFN and RBV were not toxic to the cells at the indicated concentrations. As shown in Figures 4 and 5, the inhibition of HCV RNA replication occurred in a dose-dependent manner with IFN or RBV treatments. RBV at a concentration of 100 μM inhibited replication of RNA (Fig. 5), but was not toxic to OR6c cells (Fig. 6).

The inhibitory effect of 100 μM RBV on RNA replication in each mutant was also examined. Various biological effect of IFN has been investigated and its effect on cell cycle or cell-differentiation is strong, and we focused on the effect of mutants on RBV treatment. To see this effect, we compared between IFN alone and IFN + RBV. As shown in Figure 7, no difference between three mutants was seen in the treatment with 1 unit/ml of IFN. The proliferation of each mutant RNA was similarly reduced to around a ratio of 0.6. On the other hand, addition of 100 μM of RBV was differently affected by each mutation pattern (Fig. 8). The single mutant with V85I and double mutants with V85I and K124E

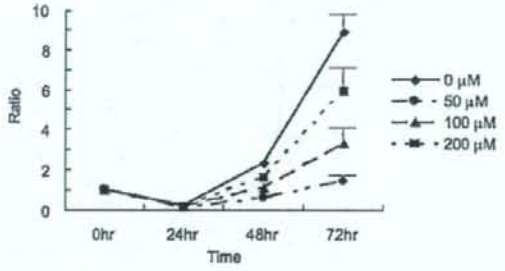

Fig. 5. Dose-dependent inhibition of HCV RNA replication by ribavirin. OR6 cells were transfected with wild-type replicon (OR/3-5B/KE) and treated with ribavirin at concentrations of 50, 100, and 200 μM for 72 hr. Inhibition of HCV RNA replication in the OR6c cell treated with ribavirin (RBV) was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

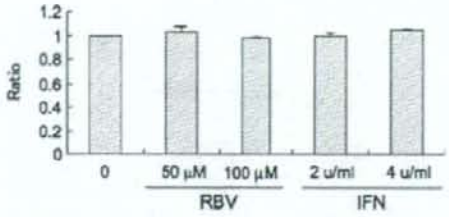

Fig. 6. Cytotoxicity of ribavirin (RBV) or interferon-α (IFN) on replicon RNA in OR6c cells. OR6c cells with OR/3-5B/KE RNA were cultured in the absence or presence of RBV or IFN (50 and 100 μM or 2 and 4 u/ml) for 72 hr, and then the cell viability was determined as described in Materials and Methods Section. The relative cell viability (%) calculated at each point, when viability of non-treated cells was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

were significantly increased in RNA proliferation. The degree of inhibition by RBV in OR/3-5B/KE(V85I) and OR/3-5B/KE(V85I&K124E) was significantly lower than that in OR/3-5B/KE, although the difference of OR/3-5B/KE(K124E) was not significant.

Indirect Immunofluorescence

To confirm the presence of replicating full-length RNAs in cells selected for G418 resistance following transfection with ON/C-5B/KE(V85I), one G418-resistant cell colony was selected at random and clonally cultured. We confirmed HCV protein expression by indirect immunofluorescence imaging and observed core protein in the replicon cells (OR6) (Fig. 9c), HCV core protein was demonstrated in the clonally isolated cell line selected after transfection with ON/C-5B/KE(V85I)

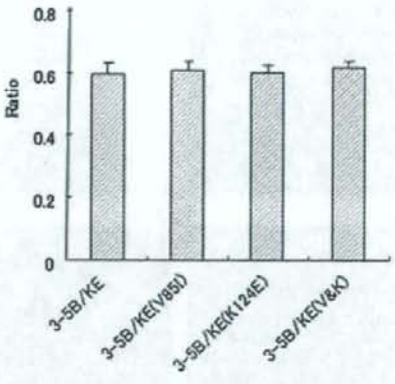

Fig. 7. Effect of interferon-α (IFN) on the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 0 u/ml or IFN 1 u/ml for 72 hr. Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase unit with IFN (1 u/ml) treatment were calculated, where the luciferase unit without IFN treatment was assigned to be 1, and compared between wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from two independent experiments.

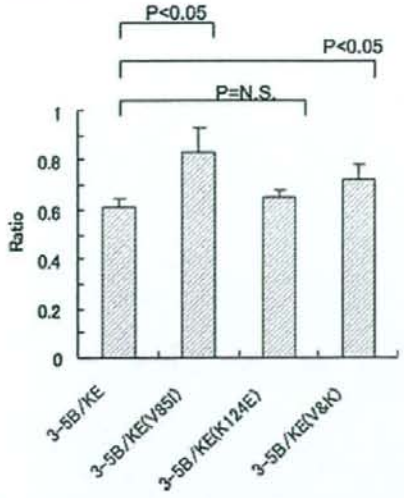

Fig. 8. Effect of interferon-α (IFN) and ribavirin (RBV) combination treatment on the replication levels of the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 1 u/ml or IFN 1 u/ml and ribavirin 100 μM for 72 hr. The relative luciferase unit of IFN 1 u/ml and ribavirin 100 mM treatment were calculated, where the luciferase unit of IFN 1 u/ml treatment was assigned to be 1, and compared in wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from three independent experiments.

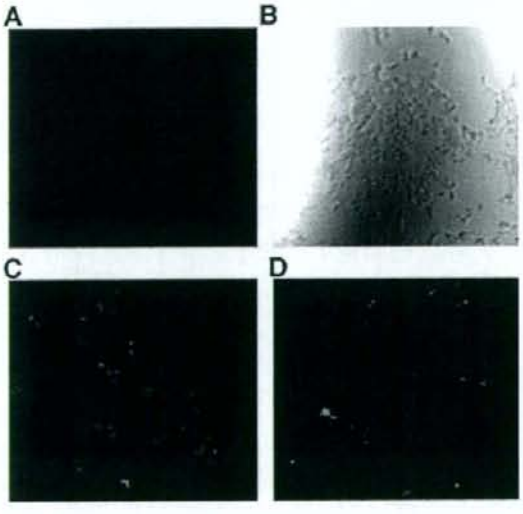

Fig. 9. Indirect immunofluorescence detection of HCV core antigen in normal OR6c cells (cured cell) (**a**), OR6 cells (wild-type HCV replicon) (**c**), and a clonally isolated cell line selected following transfection of OR6c cells with ON/3-5B/V85I (cell line 1) (**d**) and the correspondent phase-contrast microscopic photograph of OR6c cells (**b**).

(Fig. 9d), while it was not observed in the cured cell line (OR6C) (Fig. 9a,b).

DISCUSSION

Predictive factors for a sustained viral response (SVR) in IFN monotherapy or combination therapy have been vigorously investigated in prior studies. In addition to several host and viral factors, such as HCV genotypes, baseline viral load, stage of fibrosis, gender, age, and obesity [Saito et al., 2000, 2006], disappearance of serum HCV RNA during the early phase of therapy or a rapid decrease in HCV RNA levels are significant factors for achieving a SVR [Ferenci et al., 2001]. In our previous study, two distinct amino acid substitutions in the NS5B region of the HCV genome correlated with early viral responses in combination therapy [Kumagai et al., 2004]. NS5B of the HCV genome codes for RdRP, which regulates viral replication. Thus, the detected mutations might increase replication efficacy of HCV or induce resistance to the anti-viral effect of RBV, which could lead to resistance to therapy in the early phase. It was thought that the HCV replicon system would be a good tool for examining the correlation between viral mutation and replication capability. One of the mutation-introduced replicons (V85I) showed a higher replication activity than that of the wild type, and, consistent with our previous clinical study, this mutant was resistant to in vitro RBV treatment. The present study is the first to examine the precise relationship between such mutations and clinical data on the early clearance of HCV during IFN and RBV combination therapy. The mutations of V85I and K124E in NS5B have never been reported in the replicon system.

We investigated the effect of both IFN and RBV on the wild type and three mutants in NS5B at non-toxic concentration to the host cell (Fig. 6). One unit of IFN did not affect the replication of mutants (Fig. 7) but RBV significantly affected the replication of three mutants in the presence of IFN (Fig. 8). These results indicated that the polymorphism of NS5B affect sensitivity to RBV treatment. Although it has been known in the clinical setting that HCV RNA levels are not changed in patients with chronic hepatitis C during RBV monotherapy, our in vitro results showed the reduction of HCV RNA replication with RBV treatment. It was reported that serum levels of RBV in patients with chronic hepatitis C under IFN + RBV combination therapy was very low such as 10^{-14} mM [Naka et al., 2005], however, we can examine the anti-viral effect of much higher levels of RBV on the replicon system without a direct toxic effect of RBV in HuH7 cells. The possibility of a difference between circulating HCV particles and the replicon system in terms of RBV sensitivity may still exist, but this question will be further investigated using a recently developed cell culture system.

We used a dicistronic genome length and subgenome HCV RNA replication systems, which were established previously using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. For the

cells into which genome-length and subgenomic HCV RNA were introduced, we chose the cloned cell line OR6c, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since OR6c had a higher efficiency of colony formation (ECF) than its parental HuH-7 cell line in a study of subgenomic HCV replicons [Blight et al., 2002]. It is known that the efficiency of colony formation is unstable, so that the luciferase activity and the colony-forming unit are always discrepant. The impact of ON/C-5B/KE(V85I) on colony formation was about 4 times that of the wild-type replicon in genome length and subgenomic RNAs, and the V85I mutation in NS5B showed 1.5 times higher replication activity in luciferase assay than the wild type in the subgenomic replicon system. Young et al. reported an RBV-resistant NS5B mutation during RBV monotherapy [Young et al., 2003], but this phenylalanine to tyrosine amino acid substitution located at the 415th position in NS5B differed from our amino acid substitution. Replicon cells were selected after G418 exposure, and the replication may be amplified by this selection culture. We sequenced the NS5B region, which includes the 85th and 124th nucleotide portions, from some clones 2 months after G418 selection culture, and we did not found significant mutations. From the present in vitro study and previous clinical study, it may be concluded that at least V85I mutation in NS5B increases viral replication that may cause resistance to RBV treatment.

Two of the patients in the clinical study [Kumagai et al., 2004] had previously been treated with IFN-α monotherapy in our previous study: one patient (Pt 3) has V85 and K124 in the HCV RdRP and the other (Pt 7) had I85 and E124. The former was a good responder to IFN-α and RBV combination therapy, but the latter was not. This result indicated that I85V and E124K substitutions did not affect the response to IFN-α monotherapy, because both types had failed to respond previously to IFN monotherapy. Therefore, we surmised that this amino acid substitution influenced the response to RBV anti-viral activity, which prompted us to examine the effect of RBV on viral replication. Several mechanisms of anti-viral activity of RBV have been proposed [Tam et al., 1999; Maag et al., 2001; Lau et al., 2002], but it is unclear why only the V85I single amino acid substitution induced replication better than the wild type. As shown previously [Kumagai et al., 2004], the 85th amino acid of HCV RdRP is distant from the active site of polymerase but is located near the RNA primer binding site, and this substitution may influence nucleotide misincorporation during polymerization. This 85th position is more important than the 124th position for replication of HCV-O.

This study is the first to examine whether NS5B polymorphism affects the replication efficiency and anti-HCV effect of RBV in an HCV RNA replicon system. It will be interesting to know whether these mutations in other genotypes (genotypes 2 and 3) replicate more efficiently and are more resistant than genotype 1b to RBV alone. Our data suggested that during clinical use of RBV, several mutations in the HCV genome might occur, such as in the isoleucine residue at the 85th position of HCV NS5B, which then affect viral replication and RBV resistance. This viral mutation may be one of the reasons for the failure in early viral clearance by IFN and RBV. There are, however, many factors that influence the success of IFN and RBV combination therapy. The resistance or sensitivity to IFN or peg-IFN, not to RBV, might also affect the early viral response, and many factors in both viral and host sides are known to affect IFN responsiveness, such as NS5A mutations [Enomoto et al., 1996], immunological status [Saito et al., 2000], or irf-1 gene promoter polymorphisms [Saito et al., 2002, 2005]. Together, these factors might determine the efficacy of anti-viral therapy in vivo, and the present in vitro data provides evidence partially supporting our clinical observations that NS5B polymorphisms are associated with early viral clearance during IFN and RBV therapy. However, it is unclear whether this single mutation occurs with peg-IFN plus RBV combination therapy and further studies are necessary. Nevertheless, our report is useful for modeling targets for antiviral compounds for the treatment of HCV.

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Impaired Cytokine Response in Myeloid Dendritic Cells in Chronic Hepatitis C Virus Infection Regardless of Enhanced Expression of Toll-Like Receptors and Retinoic Acid Inducible Gene-I

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Dendritic cells utilize various sets of Toll-like receptors (TLR) or cytosolic sensors to detect pathogens and evoke immune responses. In patients with hepatitis C virus (HCV) infection, a higher prevalence of various infectious diseases is reported; suggesting that innate immunity against pathogens is impaired. The aim of this study was to clarify whether the TLR and retinoic acid inducible gene-I (RIG-I) system in myeloid dendritic cells is preserved or not in chronic HCV infection. The expression of TLRs, RIG-I and its relatives were compared in myeloid dendritic cells between 39 patients and 52 healthy volunteers. The induction of type-I interferon (IFN) and inflammatory cytokines was examined in response to agonists for TLR2 (palmitoyl-3-cysteine-serine-lysine-4), TLR3/RIG-I (polyinosine–polycytidylic acid) or TLR4 (lipopolysaccharide). The relative expressions of TLR2, TLR4, RIG-I, and LGP2 from the patients were significantly higher than those from the volunteers, whereas TLR3 and MDA-5 expressions did not differ. In search for factors regulating TLR/RIG-I expression, it was shown that IFN-α, polyinosine–polycytidylic acid and lipopolysaccharide induced TLR3, TLR4 and RIG-I, but TNF-α, HCV core or HCV non-structural proteins did not. For the functional analyses, myeloid dendritic cells from the patients induced significantly less amounts of IFN-β, TNF-α and IL-12p70 in response to polyinosine–polycytidylic acid or lipopolysaccharide. It is noteworthy that the expression of TRIF and TRAF6, which are essential adaptor molecules transmitting TLR3 or TLR4-dependent signals, is reduced in the patients. Thus, innate cytokine responses in myeloid dendritic cells are impaired regardless of enhanced expressions of TLR2, TLR4, and RIG-I in HCV infection. ***J. Med. Virol. 80: 980–988, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; myeloid dendritic cell; innate immunity; TLR3; RIG-I

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus, which causes chronic liver disease in hosts. At primary HCV infection, approximately 80% of patients fail to eradicate HCV and eventually progress to a chronic infected state [Lauer and Walker, 2001]. It is very likely that escape mutation of the HCV genome and insufficient immune responses against HCV in hosts are involved in the persistence of infection, however, the precise mechanisms are still largely unknown. Type-I interferon (IFN) is a potent anti-viral agent that exerts its ability by suppressing viral replication or via modulating immune reactions. Gene expression analyses of HCV-infected livers obtained from chimpanzees revealed that type-I IFN and IFN-stimulated genes are highly induced even in the incubation phase [Bigger et al., 2004]. Nevertheless, HCV continues to replicate and remains at high titer levels, suggesting that HCV

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possesses some inhibitory mechanisms in IFN-inducible anti-viral responses.

As for the mechanisms of HCV persistence, the alteration or impairment of various immune cells has been reported, such as T cells, NK cells and dendritic cells [Chang et al., 2001; Wedemeyer et al., 2002; Kanto et al., 2004; Szabo and Dolganiuc, 2005]. In clear contrast with the human immunodeficiency virus, HCV does not lead to generalized immune suppression in infected hosts. Large-scale epidemiological study on US veterans revealed that the prevalence of various infectious diseases was significantly higher in HCV-positive individuals than in HCV-negative ones, including viral, bacterial, and parasite diseases [El-Serag et al., 2003]. These observations suggest that HCV infection raises the susceptibility to pathogens, not profoundly but significantly, in infected patients. However, the underlying mechanisms in the increased prevalence of infection are yet to be determined.

Toll-like receptors (TLR) are expressed in epithelial cells or antigen presenting cells and act as sensors of bacterial or viral infection. These cells utilize specific TLR for the recognition of pathogen-associated molecular patterns and eventually induce type I IFN or inflammatory cytokines. In addition to the TLR system, the existence of cytoplasmic receptors for dsRNA has been reported as virus sensors, which are retinoic acid inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) [Yoneyama et al., 2004]. Since dsRNA is a replicative intermediate of RNA virus, RIG-I and MDA-5 induce IFN-β in response to virus infection independently of TLR3. It is thus plausible that a disabled TLR/RIG-I system may be involved in the increased susceptibility to pathogens or the mechanisms of persistent virus infection [Sumpter et al., 2005]. In human hepatoma cells harboring HCV replicons, it has been shown that HCV NS3/4A protease impedes TLR3-dependent or RIG-I-dependent IFN-β induction by means of the cleavage of relevant adaptor molecules, such as TIR domain-containing adapter inducing IFN-β (TRIF) or interferon-β promoter stimulator-1 (IPS-1), respectively [Foy et al., 2005; Li et al., 2005]. However, it is not clear whether similar inhibitory machinery of HCV operates or not in immune cells, such as dendritic cells.

Dendritic cells are immune sentinels that play a central role against pathogens in inducing innate as well as adaptive immune responses. Dendritic cells consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of immune responses. Dendritic cells utilize various sets of TLR or RIG-I/MDA-5 to sense virus infection. After the recognition, dendritic cells begin to mature and gain the ability to produce type-I IFN and inflammatory cytokines. It has been reported that blood dendritic cells expresses distinct profiles of TLRs; human myeloid dendritic cells express TLR2, -3, -4, -5, -6, -7, and -8, while plasmacytoid dendritic cells express TLR7, -8 and -9 [Iwasaki and Medzhitov, 2004]. Numerical and/or functional impairment of blood dendritic cells in acute or chronic HCV infection has been reported by several investigators including us [Kanto et al., 2004; Szabo and Dolganiuc, 2005]. One of the plausible mechanisms leading to dendritic cells impairment may be direct HCV infection to blood dendritic cells or their precursors. In support for this, it was shown that myeloid dendritic cells are susceptible to HCV infection, judging from the results of an inoculation study with pseudo-HCV particles or detection of negative strand HCV-RNA [Kaimori et al., 2004]. According to another report, myeloid dendritic cells displayed impaired expression of IL-12 and TNF-α in response to poly-inosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) in patients with a large amount of cell-associated HCV [Rodrigue-Gervais et al., 2007], suggesting a possible link between direct HCV infection to myeloid dendritic cells and an impaired innate response.

Taking these reports into consideration, the current study focused on myeloid dendritic cells in order to clarify the roles of the TLR/RIG-I system in HCV infection, by comparing the expression of TLR, RIG-I, and MDA-5 and the induction of cytokines in response to specific agonists for these virus sensors. The study demonstrated that myeloid dendritic cells from HCV-infected patients induces a significantly lesser amount of cytokines in spite of enhanced expressions of TLR2, TLR4, and RIG-I. These findings imply that alteration of the TLR/RIG-I system is instrumental in impairment of innate immunity in HCV infection, where myeloid dendritic cells play a key role as immune sentinels against pathogens.

MATERIALS AND METHODS

Subjects

Thirty-nine patients (male/female: 22/17, mean age: 53.4 ± 10.3 years old, mean serum ALT levels: 93.9 ± 51.0 IU/L, HCV serotype 1/serotype 2: 39/0) with chronic hepatitis C (HCV group) followed at Osaka University Hospital (Osaka, Japan) were enrolled in the present study. All of them were confirmed to be positive for both serum anti-HCV antibody and HCV RNA (mean HCV RNA quantity assayed by Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics, Tokyo, Japan; [Pawlotsky et al., 2000]: $1,637 \pm 402$ KIU/ml) but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus (HIV). The presence of other liver diseases, such as alcoholic, metabolic or autoimmune hepatitis, was ruled out. Thirteen patients with chronic HBV infection determined by serum HBsAg-positive and ALT abnormality (male/female: 6/7, HBeAg+/HBeAg−: 7/6, mean age: 45.9 ± 14.4 years old, mean serum ALT levels: 95.2 ± 145 IU/L, mean HBV-DNA levels assayed by Cobas Amplicor HBV monitor Roche Diagnostics; [Noborg et al., 1999]: 6.1 ± 1.7 $\log_{10}$ copies/ml) were also enrolled as disease controls (HBV group). The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each patient. The

controls were 52 healthy volunteers or blood donors (healthy donors group) at the Osaka Red Cross Blood Center (Osaka, Japan), who were confirmed to be negative for HCV, HBV, and HIV. The background data of the blood donors were not accessible due to the confidentiality regulations of the blood center, but their serum ALT levels were confirmed to be within the normal range.

Reagents

Palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) was purchased from InvivoGen (San Diego, CA). Polyinosine–polycytidylic acid (polyI:C) and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO). Recombinant human IL-6, IL-10, and IL-12 were purchased from InvivoGen. Recombinant TNF-α was purchased from Genzyme (Framingham, MA). Recombinant HCV structural or non-structural (NS) proteins expressed by *E. coli* were purchased from Virogen (Watertown, MA). They were HCV core (amino acid positions, from 2 to 192), NS3 (from 1,450 to 1,643), and NS4 (from 1,658 to 1,863), respectively. HCV NS5B protein (from 2,421 to 2,965) was kindly provided by Japan Tobacco Corp. (Tokyo, Japan). Natural human interferon-α was purchased from Otsuka Pharmaceutical Co. (Tokyo, Japan).

Isolation of Myeloid Dendritic Cells

Peripheral blood mononuclear cells were isolated from heparinized venous blood by centrifugation on Ficoll-Hypaque cushion as described previously [Kanto et al., 2004]. Myeloid dendritic cells were magnetically isolated using a BDCA-1 Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of myeloid dendritic cells (Lineage-negative, $HLA\text{-}DR^+$, $CD11c^+$, and $CD123^{dim+}$ cells) was more than 95% as assessed by FACS (data not shown). Short-term culture of myeloid dendritic cells was performed in cytokine-free Isocove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L non-essential amino acid at 37°C in 5% CO_2.

To clarify the factors influencing the expressions of TLR or RIG-I in myeloid dendritic cells, fresh myeloid dendritic cells obtained from uninfected controls were incubated for 2 hr in the presence or absence of various cytokines, agonists for TLR/RIG-I or recombinant HCV proteins. After the incubation, they were subjected to RT-PCR analyses for the comparison.

In order to compare the function of TLR/RIG-I-mediated responses in myeloid dendritic cells between the groups, myeloid dendritic cells were incubated with various agonists for 2 hr and subjected them to cytokine analysis by RT-PCR. Alternatively, myeloid dendritic cells were cultured in the presence or absence of 25 μg/ml of polyI:C for 24 hr and collected supernatants for subsequent cytokine analyses.

Flowcytometric Analysis

The phenotypes of myeloid dendritic cells were analyzed using FACS Calibur and CellQuest software (BD Biosciences, San Jose, CA). For the staining, myeloid dendritic cells were incubated with specific antibodies for 15 min at room temperature in phosphate buffered saline (PBS) containing 2% of bovine serum albumin and 0.1% of sodium azide. The following FITC-, PE-, or APC-conjugated anti-human monoclonal antibodies were used: CD11c (clone, B-ly6), HLA-DR (L243), CD80 (L307.4), CD86 (IT2.2), CD40 (5C3), and CD83 (HB15e). All were purchased from BD Biosciences.

Real-Time Quantitative PCR

Total RNA was extracted from more than 10^6 myeloid dendritic cells using RNeasy Mini kit (Qiagen, Hilden, Germany), which was subsequently reverse transcribed in 20 μl volume using SuperScript III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Random hexamers were added as primers. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). For the quantification of TLR2, TLR3, TLR4, RIG-I, MDA-5, LGP2, myeloid differentiation factor 88 (MyD88), IPS-1, TRIF, TNF receptor associated factor 6 (TRAF6), TNF-α and IFN-β, ready-to-use assays (Taqman Gene Expression Assays, Applied Biosystems) were utilized, according to the manufacturer's instructions. All of the reagents used for PCR were purchased from Applied Biosystems. All of the reactions were performed in duplicate. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A calibrator sample from healthy volunteers was identified. The expressions of molecule were expressed as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, β-actin mRNA from each sample was quantified as a control of internal RNA and corrected all values with this.

Enzyme-Linked Immunosorbent Assay and Cytokine Beads Assay

The quantity of IFN-α in culture supernatants was evaluated using Human Interferon Alpha ELISA kit (PBL Biomedical Laboratories, New Brunswick, NJ) according to the manufacturer's instructions. The concentration of TNF-α, IL-6, and IL-12p70 in the supernatants was assayed by the use of BD cytokine beads assay (CBA) Flex Sets (BD Biosciences) and analyzed by FACS Calibur according to the manufacture's instructions. The detection limits of IFN-α, TNF-α, IL-6, and IL-12p70 are 10–5,000 pg/ml, respectively.

Statistical Analysis

The Mann–Whitney *U*-test was performed to evaluate differences among the groups using StatView

5.0 software (SAS Institute, Cary, NC). A P-value of <0.05 was considered to be statistically significant.

RESULTS

Expressions of TLR2, TLR4, and RIG-I Were Higher in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

With respect to the phenotypes of fresh myeloid dendritic cells, the expressions of maturation markers such as CD40, CD80, CD83, and CD86 were relatively low and were not different between the HCV group and healthy donor group (Fig. 1). The similar results were obtained from HBV group (data not shown). These results show that myeloid dendritic cells from all groups are equally immature phenotypes.

First, the expressions of TLR2, TLR3, and TLR4 in myeloid dendritic cells were examined. The relative amounts of TLR2 and TLR4 in the HCV group were higher than those in healthy donors or the HBV group (Fig. 2). In contrast, the TLR3 expression was not different among the groups (Fig. 2). In comparison between HBV and healthy donor groups, there was no difference in the expressions of these TLRs in myeloid dendritic cells (Fig. 2).

The expression of cytoplasmic receptors for dsRNA in myeloid dendritic cells was also compared. The RIG-I and LGP2 expression in the HCV or the HBV group was significantly higher than those from healthy donors, whereas MDA-5 did not differ among the groups (Fig. 2). No correlation was found among the expressions of any TLR and dsRNA receptors (data not shown).

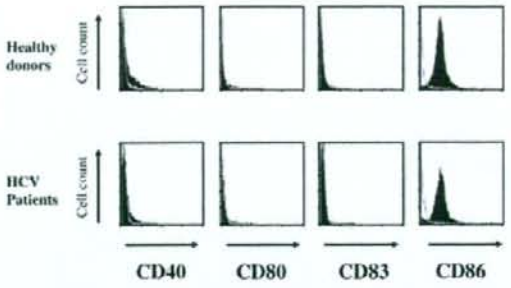

Fig. 1. Fresh myeloid dendritic cells are immature regardless of HCV infection. Myeloid dendritic cells were obtained from HCV-infective patients or healthy donors and their expressions of CD40, CD80, CD83, and CD86 were analyzed by flow cytometry. The shaded histograms are the results with specific Abs, while the open ones are those with isotype Abs. Representative results from five HCV-infected patients and five controls are shown.

IFN-α or PolyI:C Enhanced RIG-I Expression in Myeloid Dendritic Cells

To clarify the factors influencing TLR2, 3, 4, or RIG-I expression in myeloid dendritic cells, it was examined

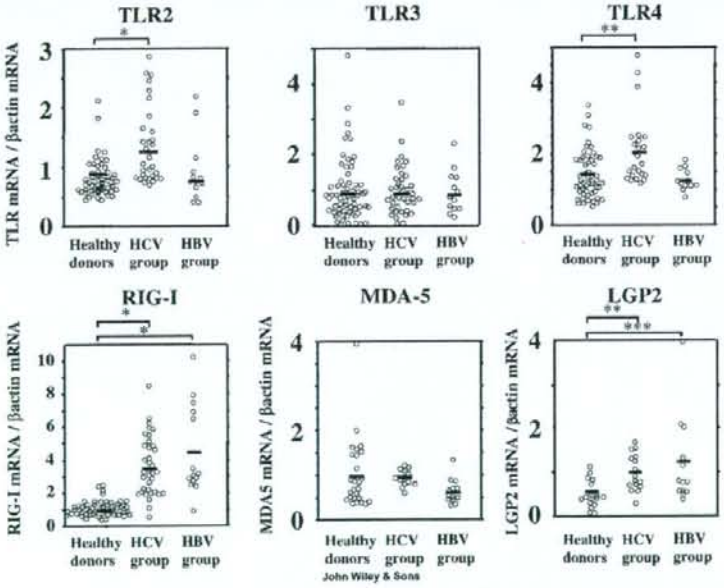

Fig. 2. Expressions of TLR2, TLR4, RIG-I, and LGP2 in patient myeloid dendritic cells from HCV-infected patients are higher than those from healthy donors, while TLR3 and MDA-5 are comparable. Expressions of TLR2, TLR3, TLR4, RIG-I, MDA-5, and LGP2 in myeloid dendritic cells were quantified by real-time RT-PCR as described in Materials and Methods Section. Horizontal bars represent the median. The statistical difference was evaluated by the Mann–Whitney U-test. $^{*}P < 0.0001$, $^{**}P < 0.0005$, $^{***}P < 0.005$.

whether they correlated with clinical parameters, such as age, serum ALT, HCV-RNA, and HBV-DNA titers. No correlation was found between any of these markers and TLR2, TLR3, TLR4, or RIG-I expressions (data not shown). Therefore, the degree of expression of these sensors is not involved in the control of virus replication or liver inflammation. Their expressions in myeloid dendritic cells cultured with and without various reagents were compared. The ratio of the quantity was determined between samples with and without treatments and their positive induction was defined as more than 2.0. The kinetics of agonist-induced TLR2, TLR3, TLR4, or RIG-I expression were preliminarily examined in myeloid dendritic cells recovered from volunteers or patients. It was found that they showed a peak at 2 hr after the stimulation, which were the same either they were HCV-infected or not (data not shown). Thus, in the following experiments, cells were obtained at this point and subsequently analyzed transcripts of target genes.

In the present study, IFN-α significantly enhanced RIG-I expression in myeloid dendritic cells (Fig. 3A). A similar effect of IFN-α was observed in TLR3 and TLR4 expression, although at much lesser degrees than those of RIG-I. In chronic hepatitis C patients, serum levels of IL-6, TNF-α, or IL-10 have been reported to be higher than those in uninfected individuals, suggesting their roles in the pathogenesis of HCV infection [Spanakis et al., 2002]. However, the addition of these cytokines or IL-12 to myeloid dendritic cell did not influence TLR or RIG-I expression (Fig. 3B). As for TLR agonists, polyI:C or LPS significantly enhanced RIG-I expression, but only slightly enhanced TLR4 (Fig. 3B). TLR2 agonist Pam_3CSK_4 did not influence the levels of TLR and RIG-I (Fig. 3B). None of the HCV proteins had a positive impact on TLR2, TLR3, TLR4, and RIG-I expressions (Fig. 3B).

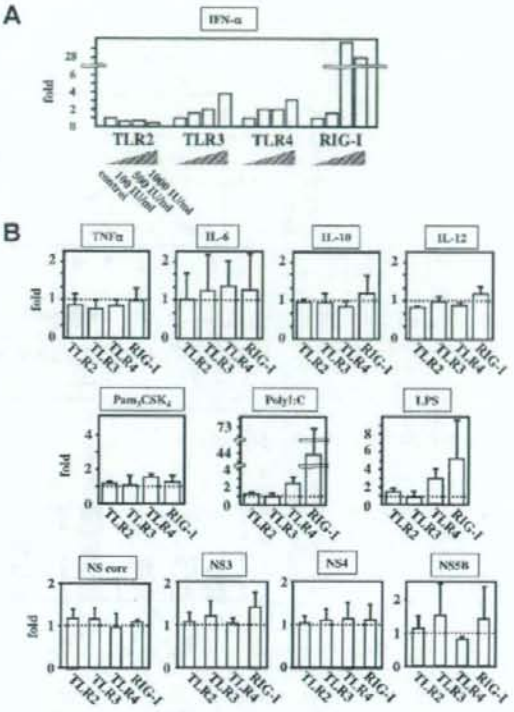

Fig. 3. IFN-α and polyI:C are inducers of TLR3, TLR4, or RIG-I in myeloid dendritic cells. **A**: Various doses of IFN-α were added to myeloid dendritic cells obtained from healthy donors and their mRNA expressions of TLR2, TLR3, TLR4, and RIG-I were quantified by real-time RT-PCR as described in Materials and Methods Section. Bars represent the mean fold increase of relevant transcripts to those of each control. Representative results from three donors are shown. **B**: Changes of TLR2, TLR3, TLR4, and RIG-I expression in myeloid dendritic cells were examined by the addition of various cytokines, TLR agonists or recombinant HCV proteins as described in Materials and Methods Section. The fold increase was determined by the ratio of each transcript of samples with reagents to those without and expressed as the mean ± SEM. The concentration of reagents were 10 ng/ml of TNF-α or IL-6, 20 ng/ml of IL-10, 200 pg/ml of IL-12, 100 ng/ml of Pam3CSK4, 25 μg/ml of polyI:C, 100 ng/ml of LPS and 2.5 μg/ml each of HCV core, NS3, NS4, and NS5B. Representative results from five donors are shown.

Induction of IFN-β, TNF-α, and IL-12 p70 With TLR Agonists Is Impaired in Myeloid Dendritic Cells From Chronic Hepatitis C Patients

First, IFN-β and TNF-α expression were examined in myeloid dendritic cells as representatives in response to specific agonists. Since the expression of these genes in myeloid dendritic cell showed a peak at 2 hr after the stimulation either they were from donors or patients (Fig. 4A), samples were collected at this point. In myeloid dendritic cells stimulated with polyI:C, IFN-β was significantly induced in the HCV, the HBV, and healthy donor groups (Fig. 4B). However, their expression from HCV or HBV-infected patients was significantly lower than that from healthy donors (Fig. 4B). Agonists for TLR3 or TLR4 significantly stimulated myeloid dendritic cells to induce TNF-α regardless of HCV or HBV infection. As the same IFN-β, TNF-α induction in myeloid dendritic cells stimulated with polyI:C or LPS was lower in the HCV or the HBV group (Fig. 4B). Therefore, in myeloid dendritic cells from hepatitis C patients, in spite of higher expression of TLR2, TLR4, and RIG-I, their levels of agonist-induced IFN-β and TNF-α were less than those in healthy donors.

To compare more precisely the cytokine response in myeloid dendritic cell between HCV-infected patients and donors, the levels of IFN-α, TNF-α, IL-6, and IL-12 p70 in supernatants were examined. Since the induction of IFN-β and TNF-α in myeloid dendritic cell was profound in the presence of polyI:C, samples were collected from myeloid dendritic cells stimulated with polyI:C. The levels of IFN-α and IL-6 were not different between the groups (Fig. 4C). In contrast, the amounts of TNF-α and IL-12 p70 from patients group were significantly lower than those from the donor group (Fig. 4C). These results suggest that some inhibitory

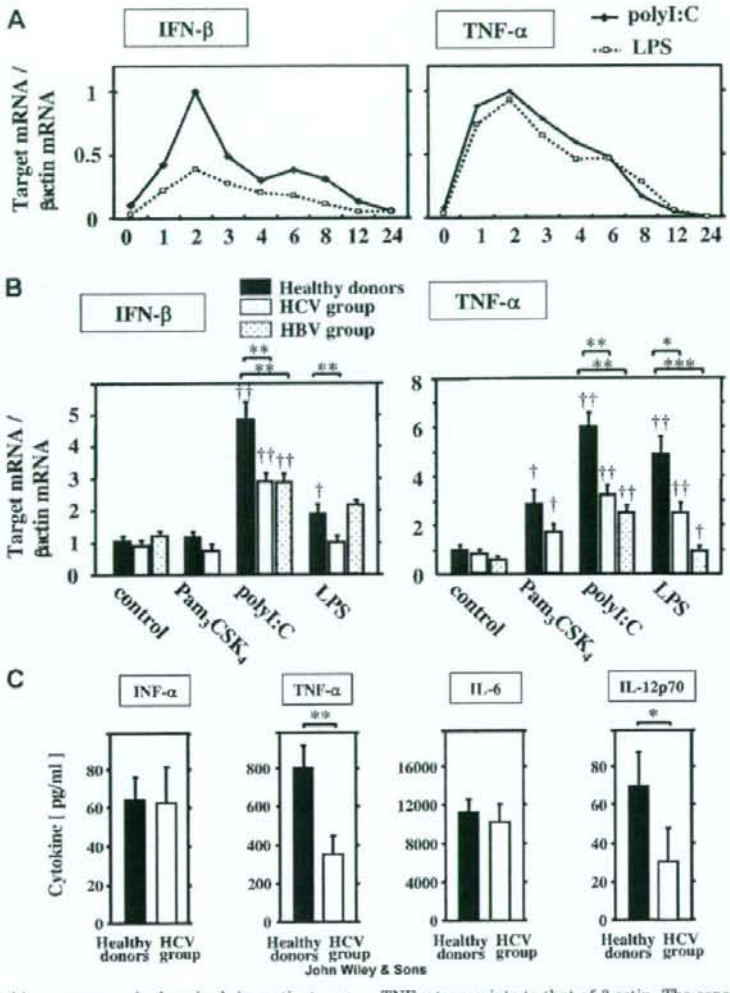

Fig. 4. Innate cytokine response is impaired in patient myeloid dendritic cells from HCV-infected patients. **A**: Kinetics of IFN-β and TNF-α in myeloid dendritic cells stimulated with polyI:C or LPS. The expressions of IFN-β and TNF-α in myeloid dendritic cells from healthy donors were quantified by real-time RT-PCR as described in Materials and Methods Section. At several time points before and after the stimulation of myeloid dendritic cell with 25 μg/ml of poly I:C or 100 ng/ml of LPS, the samples were subjected to RT-PCR analyses. The results are expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. Representative results from three healthy donors are shown. **B**: Expressions of IFN-β and TNF-α in myeloid dendritic cells stimulated with various TLR agonists were quantified by real-time RT-PCR as described in Materials and Methods Section. Two hours after the stimulation of myeloid dendritic cells with Pam_3CSK_4, polyI:C or LPS, the samples were subjected to RT-PCR analyses. The results were expressed as the ratio of IFN-β or TNF-α transcripts to that of β-actin. The concentrations of agonists were 100 ng/ml of Pam_3CSK_4, 25 μg/ml of polyI:C and 100 ng/ml of LPS. The bars represent mean + SEM. $^{\dagger}P < 0.05$ vs. control, $^{\dagger\dagger}P < 0.01$ versus control, $^{*}P < 0.05$ versus healthy donors, $^{**}P < 0.01$ versus healthy donors, $^{***}P < 0.001$ versus healthy donors. Representative results from 14 HCV-infected patients, 13 HBV-infected patients and 25 controls are shown. Statistical differences were evaluated by the Mann–Whitney *U*-test. **C**: Myeloid dendritic cells in both groups were stimulated with polyI:C for 24 hr. The supernatants were collected and the levels of IFN-α, TNF-α, IL-6, and IL-12p70 were examined by ELISA or cytokine beads assay as described in Materials and Methods Section. The bars represent mean + SEM. Statistical differences were evaluated by the Mann–Whitney *U*-test. Representative results from 11 HCV-infected patients and 17 controls are shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

mechanisms exist downstream of TLR or RIG-I in myeloid dendritic cells from the HCV-infected patients.

Expressions of TRIF and TRAF6 Were Lower in Myeloid Dendritic Cells From the HCV-Infected Patients

In order to seek the inhibitory mechanisms of TLR or RIG-I signaling in myeloid dendritic cells, the expressions of adapter molecules, MyD88, IPS-1, TRIF, or TRAF6 were compared between the HCV and donor groups. The expressions of MyD88 and IPS-1 were higher in myeloid dendritic cells from the HCV group (Fig. 5). By contrast, the levels of TRIF and TRAF6 in myeloid dendritic cells from HCV-infected patients were significantly lower than in those from healthy counterparts (Fig. 5).

DISCUSSION

The present study demonstrated that myeloid dendritic cells from HCV-infected patients express higher levels of TLR2, TLR4, and RIG-I than those from healthy subjects. Regardless of such enhanced expression, specific agonists stimulated patient myeloid dendritic cells to induce lesser degrees of IFN-β/TNF-α/IL-12 than those from the healthy counterparts. Two conclusions were reached from the current study findings: HCV enhances expression of some TLR and RIG-I in myeloid dendritic cells, but HCV impedes TLR or RIG-I-mediated cytokine responses in them. Since dendritic cells play a role as immune sentinels, such impaired cytokine response in myeloid dendritic cell may be one of the mechanisms in enhanced susceptibility to various pathogens in HCV-infected individuals as reported elsewhere [El-Serag et al., 2003].

It has been reported that TLRs are expressed in epithelial cells and immune cells, and RIG-I is ubiquitously expressed in various cells [Yoneyama et al., 2004]. However, it remains obscure how their expressions are regulated. It is generally accepted that TLR3 and RIG-I are inducible by type-I IFN [Doyle et al., 2003; Yoneyama et al., 2004]. The current study confirmed this phenomenon also in myeloid dendritic cells, since IFN-α up-regulated TLR3, TLR4, and RIG-I expression in a dose-dependent manner. Gene expression analyses revealed that HCV infection induces type-I IFN and IFN-stimulated genes in HCV-infected liver from chimpanzees or humans [Bigger et al., 2004]. One of the triggers leading to IFN production is the presence of double-strand RNA in infected tissues, which is a replicative intermediate of HCV. The current study also showed that polyI:C is a prominent inducer of RIG-I and TLR4. Since polyI:C is a synthetic mimic of double-strand RNA, its positive impact suggests that HCV replication in myeloid dendritic cells and/or subsequent IFN production may be involved in RIG-I or TLR4 induction.

Several investigators have reported that TLR2, TLR3, or TLR4 expression is enhanced in monocytes or B cells obtained from chronic hepatitis C patients, both of which are known to be susceptible to HCV [Machida et al., 2006; Riordan et al., 2006]. Regardless of the difference in cell types, the present study offers support for the enhanced TLR2 and TLR4 expression in HCV infection described by these reports. As for the mechanisms, TNF-α or HCV NS5A has been reported to be involved in TLR2 or TLR4 up-regulation [Machida et al., 2006]. However, in this study, addition of recombinant TNF-α or the HCV proteins failed to induce any TLR or RIG-I in

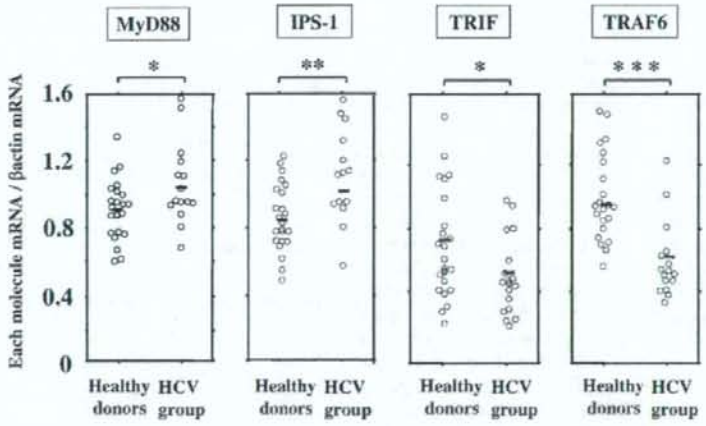

Fig. 5. Expressions of TRIF and TRAF6 are lower but those of MyD88, IPS-1 are higher in patient myeloid dendritic cells than those from healthy counterparts. Expressions of MyD88, IPS-1, TRIF TRAF6 were quantified by real-time RT-PCR as described in Materials and Methods Section. The results were expressed as the ratio of each transcript to those of β-actin. Horizontal bars represent the median. Statistical differences were evaluated by the Mann–Whitney *U*-test. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

myeloid dendritic cells. Therefore, enhanced expressions of TLR2, TLR4, and RIG-I in myeloid dendritic cells may be due to, not completely but in some part, the existence of HCV in cells or the exposure to endogenous IFN-α. To check this, it may be necessary to conduct studies with inoculation of HCV particles or transduction of the viral genome in myeloid dendritic cells.

In comparison of the results between the HCV and the HBV groups, the expressions of TLR2 and TLR4 in the HBV group were comparable with those from healthy donor group, suggesting that the induction of TLR2 and TLR4 in myeloid dendritic cells is unique in HCV infection. In contrast, the levels of RIG-I and LGP2 were comparable between the HCV and the HBV groups, both of which were higher than those from healthy donors. These results raise the possibility that, regardless of the difference of hepatitis virus, similar mechanisms may be involved in the induction of RIG-I and LGP2 in myeloid dendritic cells.In cells bearing HCV replicons, it has been reported that HCV NS3/4A inhibits TLR3 or RIG-I-mediated IFN-β induction by the cleavage of relevant adaptor molecules TRIF or IPS-1, respectively [Foy et al., 2005; Li et al., 2005]. In the present study, in myeloid dendritic cells from the HCV group, polyI:C-stimulated IFN-β, TNF-α, and IL-12 p70 induction is impaired. As for the adaptor molecules in TLR-dependent signals, TRIF and TRAF6 expression was lower in HCV-infected patients than those in healthy donors. Since it has been proven that the cleavage of TRIF hampers TLR3-mediated IFN production [Fitzgerald et al., 2003], the current study implies that lower expression of TRIF is involved in the inhibition of TLR3 or TLR4-mediated signals in myeloid dendritic cells. Of particular interest is the possibility that such reduction of TRIF and TRAF6 in myeloid dendritic cells is caused by the cleavage by NS3/4A, as shown in hepatoma cells [Foy et al., 2005; Li et al., 2005]. If this does occur, the inhibitor of NS3/4A serine protease may be able to restore TLR-dependent innate responses in myeloid dendritic cells, in addition to its potent suppressive ability of HCV replication. Machida et al. reported that enhanced expression of TLR4 in HCV-infected B cells is related to the TLR4-dependent up-regulation of IFN-β and IL-6, suggesting that TLR4-dependent signals are not impaired in B cells [Machida et al., 2006]. Further study is necessary to reveal whether HCV does actually influence innate immunity according to differences in blood cell types. In the current study, polyI:C or LPS-stimulated myeloid dendritic cells from HBV-infected patients induced lesser degree of IFN-β or TNF-α, respectively. Several investigators reported that the function of blood dendritic cells in HBV-infected patients were impaired [Tavakoli et al., 2004; van der Molen et al., 2004]. It is yet to be determined whether HBV infects to myeloid dendritic cells or not. The current study raises the possibility that distinct mechanisms are involved in the impairment of TLR or RIG-I pathway according to the difference of virus. Further study depending on expression as well as functional assay of virus recognition system in HBV infection is needed to clarify these important issues.

In contrast with RIG-I and LGP2, MDA-5 expression in myeloid dendritic cells from HCV-infected patients was comparable with that from healthy donors, suggesting that these cytosolic RNA sensors are regulated independently. Recently, it has been reported that RIG-I is expected to be involved in the detection of Flaviviridae, which HCV belong to, but MDA-5 is not [Hornung et al., 2006]. Active involvement of RIG-I in HCV infection has been reported, demonstrating that RIG-I, but not MDA-5, efficiently binds to secondary structured HCV RNA to confer induction of IFN-β [Saito et al., 2007]. In this study, although the polyI:C-stimulated cytokine response in patient myeloid dendritic cells was impeded, IPS-1 expression was higher than that in myeloid dendritic cells from the healthy donor group, suggesting a lesser possibility of IPS-1 as a cleavage target of HCV in myeloid dendritic cells. Alternatively, higher expression of LGP2 may contribute to the inhibitory machinery against RIG-I-mediated responses in myeloid dendritic cells, as reported elsewhere [Saito et al., 2007].

In summary, in myeloid dendritic cells from HCV-infected patients, innate cytokine responses were impaired regardless of the enhanced expressions of TLR2, TLR4, and RIG-I. These findings provide insights into the roles of the TLR/RIG-I system in the pathogenesis of HCV infection and their potentials as therapeutic targets for immune modulation.

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