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Identification of Hepatitis C Virus Core Domain Inducing Suppression of Allostimulatory Capacity of Dendritic Cells

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Hepatitis C virus (HCV) is remarkably efficient at establishing chronic infection. One of the reasons for this appears to be the suppression of the accessory cell function of professional antigen presenting cells. In the present study, the immunosuppressive activity of HCV protein was examined on dendritic cells (DCs) generated from mouse bone marrow progenitor cells *in vitro*. We found that the DCs forced to express HCV protein have defective allostimulatory ability. DCs expressing HCV protein were phenotypically indistinguishable from normal DCs. However, they were unable to produce IL-12 effectively when stimulated with lipopolysaccharide. The functional domain of the HCV protein essential for immunosuppression was determined using a series of NH_2-and C-terminal deletion mutants of HCV core protein. We found that amino acid residues residing between the 21st and the 40th residues from the NH_2-terminus of HCV core protein are required for immunosuppression. These findings suggest that HCV core protein suppresses the elicitation of protective Th1 responses by the inhibition of IL-12 production by DCs.

Key words: Hepatitis C virus, Dendritic cell, Interleukin-12, Allogeneic mixed lymphocyte reaction

INTRODUCTION

Hepatitis C virus (HCV) is one of the major causes of acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Cohen, 1999). Moreover, HCV is remarkably efficient at establishing chronic infection, as infection with HCV proceeds to chronicity in more than 80% of cases (Purcell, 1997).

Recent studies have shown that a weak Th1 response against HCV antigen is related with the establishment of chronic infections (Tsai *et al.*, 1997; Diepolder *et al.*, 1995; Lechmann *et al.*, 1996), although a high rate of genetic variation in the HCV gene may also facilitate evasion from immune surveillance (Lanzavecchia, 1997; Odeberg *et al.*, 1997). HCV appears to produce proteins that suppress the elicitation of the protective Th1 response, and this suppression by HCV products appears to be due to the altered function of professional antigen presenting cells, such as lymphoid dendritic cells (DCs) and macrophages. Direct transfection of the HCV structural gene into DCs have been shown to suppress the allostimulatory capacity of DCs (Hiasa *et al*, 1998). In addition, DCs generated from monocytes isolated from HCV-infected patients have been shown to be extremely low in allostimulatory capacity (Kanto *et al.*, 1999). More interestingly, the injection of recombinant vaccinia virus containing the HCV core gene into mice was shown to suppress vaccinia-specific CTL response, and increased the mortality of recipient mice (Large *et al.*, 1999). These observations show that HCV core protein suppresses the antigen presenting cell function of DCs. HCV core protein appears to suppress Th1 response by inhibiting the IL-12 and NO production of professional antigen presenting cells (Lee *et al.*, 2001).

In this study, the immunosuppressive activity of HCV core protein was examined on DCs generated from mouse bone marrow progenitor cells *in vitro*. We also determined the functional domain of the HCV core protein responsible for this immunosuppression using a series of NH_2- and C-terminal deletion mutants of the HCV core protein.

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MATERIALS AND METHOD

Cell culture

Cells were cultured in Dulbecco's modified Eagles medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 1% (v/v) nonessential amino acids(Invitrogen), and 50 μM 2-mercaptoethanol (Invitrogen) at 37°C in a 5% CO_2 atmosphere.

Plasmids containing the HCV gene

Plasmid pCMV-C980 encoding the core, E1, E2, p7, and C-terminal truncated NS2 was provided by Dr. Kuntada Shimotohno, The Institute for Virus Research, Kyoto University, Kyoto, Japan (Marusawa et al., 1999). Plasmids pCI-Neo/core 1-191, pCI-Neo/core 21-191, pCI-Neo/core 41-191, pCI-Neo/core 1-152 and pCI-Neo/core 1-173 were provided by Dr. Young Chul Sung, Pohang University of Science and Technology, South Korea.

Generation of dendritic cells

Immature DCs were generated from the BM cells of C57BL/6 mice, as described previously (Lee *et al.*, 2001). Briefly, total BM cells obtained from the femurs of mice were cultured in a 6-well plate (5×10^6/well) in culture medium (3 ml/well) supplemented with 200 U/ml of rmGM-CSF (PeproTech Inc., Rocky Hill, NJ) and 100 U/ml of rmIL-4 (PeproTech). After 2 and 4 days of culture, nonadherent cells were discarded by replacing the culture medium with fresh medium containing the same cytokines after gentle shaking. Immature DCs at day 4 were used for the transfection experiments.

Transfection and selection

The plasmids were transfected into immature DCs using FuGENE6 transfection reagent (Roche) according to the manufacturers instructions. Briefly, 1 μg of DNA in a volume of 10 μl was mixed with 3 μl of FuGENE6 transfection reagent that was pre-diluted with 100 μl of serum-free medium, and incubated for 15 min at room temperature. The mixture was then added to DC cultures ($5\text{-}7 \times 10^6$ cells/well). The selection of transfected cells and the induction of DC maturation were performed simultaneously by adding 400 μg/ml of G418 sulfate (Invitrogen) and 1 μg/ml of LPS (Difco) on the next day, and culturing the cells for an additional 2 days.

Allogeneic mixed lymphocyte reaction (MLR)

DCs were harvested, washed, irradiated with 2000 rads and then used as stimulator cells in an allogeneic MLR. Responder T cells were isolated from splenocytes of Balb/c strain of mice by passage through a nylon wool column. For allogenic MLR, 2×10^5 responder T cells were mixed with various numbers of DCs in 96-well plates, and cultured for 3 days. DNA synthesis was measured by ^{3}H-thymidine (Du Pont) incorporation (1 μCi/well) for the final 6 hrs of the culture period.

Immunocytochemistry

Cells were stained with monoclonal antibodies recognizing murine cell surface markers, as described previously (Lee *et al.*, 2001). The monoclonal antibodies, anti-CD40 (clone 3/23), anti-I-A^b (clone AF6-120.1), anti-B7-1 (clone 16-10A1), anti-B7-2 (clone GL1), and isotype-matched control antibodies were purchased from Pharmingen (San Diego, CA). For intracellular staining, cells were permeabilized with saponin (10%), and then stained with rabbit anti-HCV core antibody, which was kindly provided by Dr. K. Shimotohno (Kyoto University, Kyoto, Japan). Flow cytometric analysis was performed using a FACS Calibur (Becton-Dickinson). Dead cells were gated out using their low forward angle light scatter intensity. In most analysis, 10,000 cells were scored.

Elisa for IL-12

The IL-12 levels in the culture supernatants of stimulated DCs were measured using a commercial immunoassay kit (R & D System) that detected bioactive p70 heterodimer.

RESULTS

Expression of HCV protein by DCs

To examine the effect of the expression of HCV protein on the function of DCs, immature DCs were generated from mouse bone marrow progenitor cells by culture in the presence of GM-CSF and IL-4. Immature DCs were then transfected with pCMV-C980 plasmids. After overnight culture, transfected cells were selected and induced to differentiate by culturing them in the presence of 400 μg/ml of G418 sulfate and 1 μg/ml of LPS for 2 days. Mature DCs were harvested after gentle pipetting and washing, and were then counted. As shown in Fig. 1, no significant difference was found in the total number of cells finally recovered from the control plasmid group (pCMV) and the HCV gene-containing plasmid group (pCMV-C980), although the total number of cells in both groups was significantly lower than that of untransfected, and hence unselected, control group.

The level of expression of HCV core protein was examined by intracellular staining with rabbit anti-HCV core antibody, and is shown in Fig. 2. The expression of

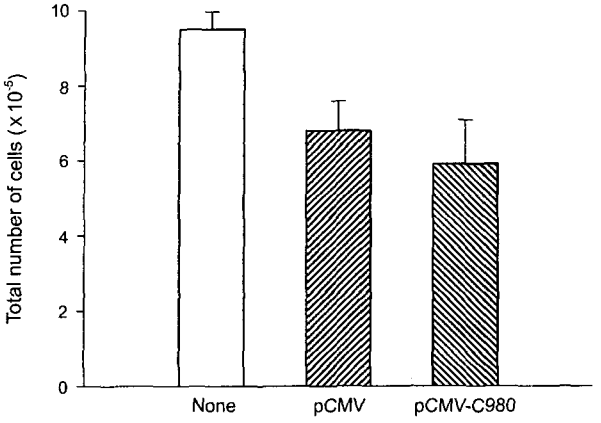

Fig. 1. Effects of DNA transfection on the generation of mature DCs. Immature DCs were generated from mouse bone marrow progenitor cells by culture in the presence of GM-CSF and IL-4. Immature DCs were then transfected with pCMV-C980 encoding the core, E1, E2, p7, and the C-terminally truncated NS2. After overnight culture, transfected cells were selected and induced to differentiate by culturing the cells in the presence of 400 μg/ml of G418 sulfate and 1 μg/ml of LPS for 2 days. Mature DCs were harvested after gentle pipetting, washed, and then counted.

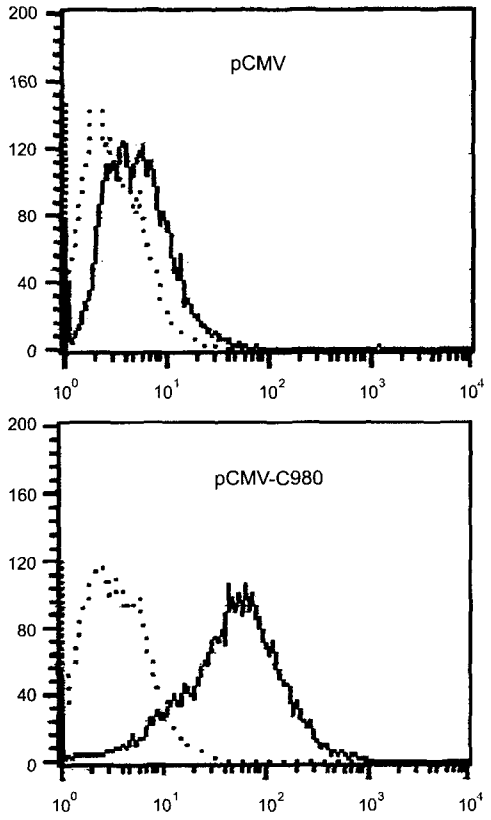

Fig. 2. Expression of HCV core antigen in DCs. The cells described in Fig. 1 were permeabilized with saponin (10%), and then stained with rabbit anti-HCV core antibody, washed, and then stained with FITC-conjuated goat anti-rabbit antibody. Flow cytometric analysis was performed on a FACS Calibur. Dead cells were gated out by their low forward angle light scatter intensity.

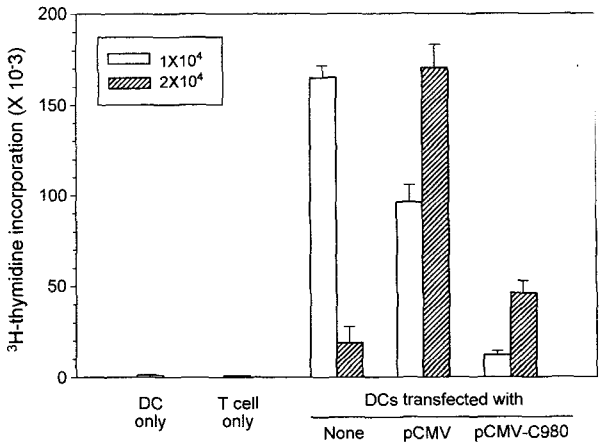

Fig. 3. Suppression of allostimulatory capacity by HCV protein. DCs were harvested, washed, irradiated with 2000 rads, and then used as stimulator cells in allogeneic mixed lymphocyte reactions. Responder T cells (2 X 10^5/well) were isolated from splenocytes of Balb/c strain of mice by passage through a nylon wool column, mixed with the indicated numbers of DCs in a 96-well plates, and cultured for 3 days. DNA synthesis was measured by ^{3}H-thymidine incorporation during the final 6 hrs of culture.

HCV core protein was observed in >90% of the DCs.

Low allostimulatory capacity of DCs expressing HCV protein

The allostimulatory capacity of the DCs was compared in a mixed lymphocyte reaction. As shown in Fig. 3, DCs expressing HCV protein (pCMV-C980) were significantly low in allostimulatory ability compared with control DCs (pCMV) that were transfected with vector itself. It is note worthy that normal DCs (none in Fig. 3) induced maximal proliferation of allogeneic T cells at a density of 1×10^4 cells/well when the degree of allogeneic T cell proliferation was measured at the indicated time point. The decreased value of ^{3}H-thymidine uptake at a higher density (2×10^4 cells/well) was due to the cease of allogeneic T cell proliferation after reaching maximal proliferation.

Phenotypic analysis of DCs expressing HCV protein

To examine whether the decreased allostimulatory activity of DCs expressing HCV protein (pCMV-C980) was due to the modulation of the expressions of cell surface molecules, the expression level of several of cell surface molecules was examined by flow cytometry. Some of the results obtained are shown in Fig. 4, which shows that the expression levels of class I MHC, class II MHC, and of co-stimulatory molecules such as B7-1, B7-2, and CD40 were not different for DCs regardless of HCV protein expression.

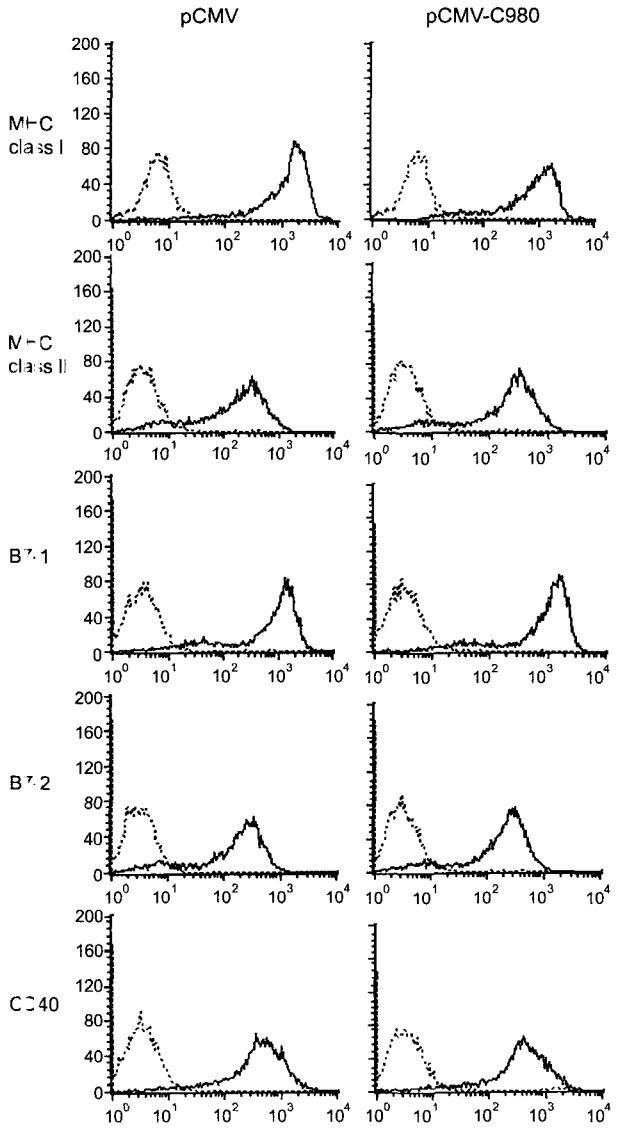

Fig. 4. Phenotypic analysis of DCs. The cells described in Fig. 1 were used for immunophenotypic analysis. Immunophenotypic profiles of DCs are shown compared to isotype controls.

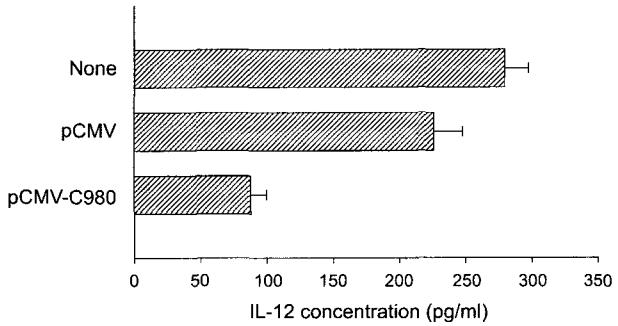

Fig. 5. Production of IL-12 by DCs. The culture supernatants of DCs stimulated with LPS were collected, and assayed for IL-12. IL-12 was measured using a commercial immunoassay kit that detected the bioactive p70 heterodimer, according to the manufacturers instructions.

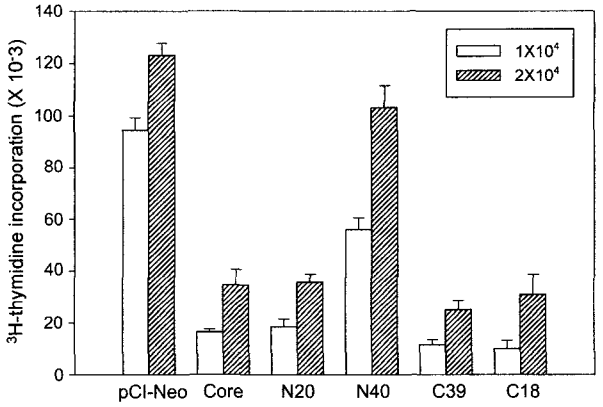

Fig. 6. Identification of the HCV core region that induces immunosuppression. Immature DCs were transfected with plasmids encoding a series of NH_2- and C-terminal deletion mutants of HCV core protein and selected as in Fig. 1. DCs expressing the whole core protein (core), those with NH_2-terminal 20 amino acids (N20) deleted, with NH_2-terminal 40 amino acids (N40) deleted, with C-terminal 39 amino acids (C39) deleted, and with C-terminal 18 amino acids (C18) deleted were used as stimulators in allogenic mixed lymphocyte reactions.

Production of IL-12 by DCs expressing HCV protein

To examine whether the decreased allostimulatory activity of DCs expressing HCV protein was due to their inability to produce IL-12, we collected the culture supernatant from the MLR cultures, and quantified the amount of bioactive IL-12 p70. However, the IL-12 p70 levels were near the quantitative limit of the assay kit. Thus, we decided to compare the ability of DCs to produce IL-12 after stimulation with LPS. As shown in Fig. 5, DCs expressing HCV protein (pCMV-C980) produced significantly smaller amounts of IL-12 p70 compared with control DCs transfected with control vector.

Identification of the core region that induces immunosuppression

The functional domain of HCV protein essential for immunosuppression was identified using a series of NH_2- and C-terminal deletion mutants of the HCV core protein. As shown in Fig. 6, DCs expressing the whole core protein (core) were defective in allostimulatory activity compared with control DCs (pCI-Neo). DCs expressing HCV core proteins with deleted NH_2-terminal 20 amino acids (N20), or C-terminal 39 (C39) or 18 (C18) amino acids were also defective in allostimulatory activity. However, DCs expressing HCV core proteins with deleted NH_2-terminal 40 amino acids (N40) exhibited significantly

increased allostimulatory activity. These results show that the amino acid residues located in between the 21st and the 40th residues from the NH_2-terminus of the HCV core protein are critical for the down-regulation of the allostimulatory activity of DCs.

DISCUSSION

The present study demonstrates that DCs forced to express HCV core protein are defective in terms of their allostimulatory ability. In addition, the present study demonstrates that the functional domain of the HCV core protein essential for immunosuppression is located between the 21st and 40th amino acids from the NH_2-terminus.

DCs are the most important antigen presenting cells (Banchereau *et al.*, 2000). They can acquire and process antigens at the periphery, migrate to secondary lymphoid tissues, and even activate naïve T cells in a primary response. DCs residing in tissues and organs of the body, however, do not express high levels of costimulatory molecules, or secrete cytokines such as IL-12, and are poor T-cell stimulators (Banchereau *et al.*, 2000). Thus, the DCs residing in the tissues and organs of the body are sometimes referred to as immature DCs (Austyn, 1996; Granucci *et al.*, 1999). These immature DCs must mature or be activated to enable them to fully perform accessory cell functions. LPS is one of the well characterized maturation-inducing agents of immature DCs (Aiba *et al.*, 1998; Manome *et al.*, 1999). The DCs used in the present study were generated from mouse BM cells by culture in a medium supplemented with GM-CSF and IL-4, and were shown to be immature (Lee *et al.*, 2001). We transfected plasmids encoding the HCV antigen into immature DCs, and the transfectant cells were then induced to further differentiate by LPS. When we examined the functional properties of the resultant DCs, we found that the DCs were severely suppressed in terms of their allostimulatory activity. One of the mechanisms of these suppressed allostimulatory ability appears to be associated with the suppression of IL-12 production by DCs. It should be noted that DCs expressing HCV protein were phenotypically indistinguishable from normal DCs.

Our results are in line with earlier observations that showed that macrophages expressing HCV core antigen are defective in terms of IL-12 and NO production (Lee *et al.*, 2001). Earlier studies have also shown that the infection of DCs isolated from mouse spleen with adenovirus-vector containing HCV structural gene causes the suppression of allostimulatory activity, which is probably due to the inhibition of IL-12 production by the DCs (Hiasa *et al.*, 1998). In addition, it was reported that DCs generated from monocytes that were isolated from HCV-infected individuals were suppressed in terms of allostimulatory activity (Kanto *et al.*, 1999). The *in vivo* relevance of our observations that demonstrated that DCs forced to express HCV core antigen have suppressed accessory cellular functions might be questioned. There is, however, evidence that mononuclear cells can be infected by HCV and that mitogenic stimulation of the infected cells increases the transcription of HCV RNA (Moldvay *et al.* 1994, 1994; Lerat *et al.*, 1996).

To identify the functional domain of the HCV core protein that induces the suppression of the accessory cell function of DCs, we transfected DCs with plasmids encoding a series of NH_2- and C-terminal deletion mutants of HCV core protein. We found that the region of the HCV core protein that is essential for immunosuppression is located between the 21st and 40th amino acids from the NH_2-terminus. This observation is consistent with an earlier report which demonstrated that the region between amino acids 21 to 40 of the HCV core protein is required for the downregulation of IL-12 and NO production in macrophages (Sung, 1999).

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