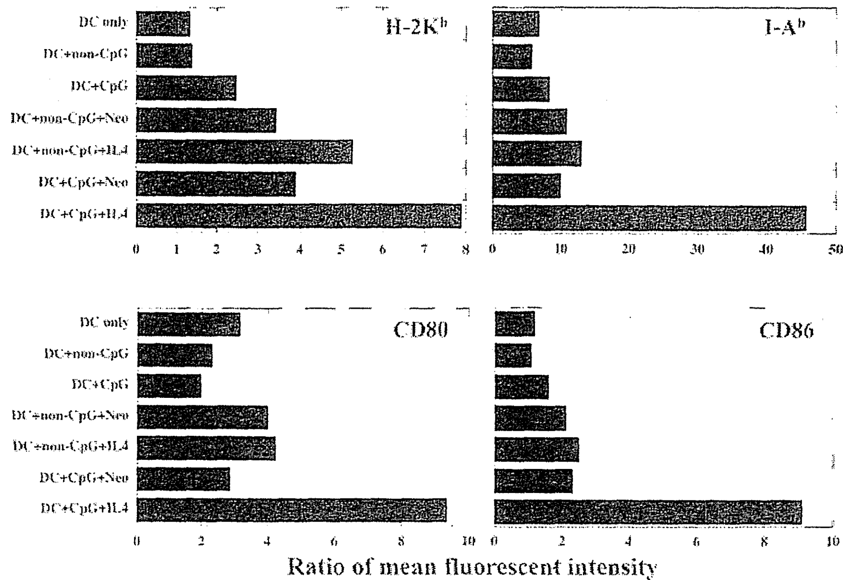

Figure 1. Coincubation with interleukin (IL)-4 and CpG-oligodeoxynucleotides (ODN) induces DC maturation *in vitro*. We incubated bone marrow-derived DCs with irradiated IL-4-overexpressing or control gene (Neo)-transduced MC38 cells either alone or in combination with CpG-ODN-1826 or non-CpG-ODN-1911 *in vitro*. Seven groups were compared: i) DC only, ii) DC + non-CpG, iii) DC + CpG, iv) DC + non-CpG + MC38-Neo, v) DC + non-CpG + MC38-IL4, vi) DC + CpG + MC38-Neo, and vii) DC + CpG + MC38-IL4. After a 24-h incubation, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-H-2K^b, I-A^b, CD80, and CD86 monoclonal antibodies. The results are shown as ratios of mean fluorescent intensity (MFI) of the incubated DCs to the MFI of FITC-conjugated control IgG of each group.

manner. Tumor size was measured twice a week using vernier calipers. Each experiment involved 4-6 mice per group. Mice with ulcerated tumors or tumors >20 mm in diameter were sacrificed. Experiments with the therapeutic model were performed twice.

Immunohistological analysis. For leukocyte detection in tumor tissues, B6 mice were injected s.c. with 3x10^5 MC38-Neo or -IL4 cells with or without 30 μg/mouse of CpG-ODN-1826 in the area surrounding the established wild-type tumor 7 days after inoculation with 3x10^5 MC38-WT cells. Tumor tissues, which were harvested 3 days after inoculation with the genetically modified tumor. Serial 5-μm sections were exposed to anti-Gr-1, anti-CD11c, anti-CD4, and anti-CD8a antibodies (Nippon Becton-Dickinson). Rat IgG2a was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Immunoreactive cells were counted in 6 fields in a light microscope (magnification, x400) in a blinded fashion.

Induction of tumor-specific CTL. We assessed the tumor-specific cytolytic activity of immune mice. Mice were initially immunized with MC38-IL4 cells in combination with 30 μg of either CpG-ODN-1826 or non-CpG-ODN-1911 on Day 0, and they were then inoculated with 3x10^5 MC38-WT cells on Day 7. Subsequently, MC38-immune mice received a challenge of 1x10^6 MC38-WT cells on Day 28. Splenocytes (3x10^6 cells/ml) were harvested from these mice on Day 42, and they were then stimulated *in vitro* with irradiated (100 Gy) MC38-IL4 tumor cells (3x10^5 cells/ml) and 6 μg/ml of non-CpG-ODN-1911 (IL-4 group) or IL4 cells in combination with 6 μg/ml of CpG-ODN-1826 (IL-4 + CpG group). Seven days later, responder cells (1x10^6 cells/ml) were restimulated with irradiated-IL4 tumor cells either alone (IL-4 group) or in combination with 6 μg/ml of CpG-ODN-1826 (IL-4 + CpG group) that was supplemented with irradiated syngeneic naive splenocytes (30 Gy, 1x10^6 cells/ml) as well as 50 IU/ml recombinant mouse IL-2 (Nippon Becton-Dickinson). Cytolytic assays were performed 6 days after the last stimulation using the responder cells as effector cells. Naïve splenocytes that were stimulated twice *in vitro* with irradiated-IL4 tumor cells and CpG-ODN as described above were used as control effector cells.

Cytolytic assays. Cytolytic assays were performed as previously described (22). Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT and YAC-1 cells, which are sensitive to non-specific killing, in triplicate in 4-h ^{51}Cr-release assays. The percentage of lysis was determined using the following formula: (release in assay - spontaneous release) x 100/(maximum release - spontaneous release). Maximum release was determined by the lysis of labeled target cells with 1% Triton X-100. Spontaneous release, which was measured by incubating target cells in the absence of effector cells, was <10% of maximum release.

Statistical analyses. Significance was assessed by Student's t-tests. Differences between groups were considered significant when the P-value was <0.05.

Results

Maturation of DCs was induced by coincubation with IL-4-overexpressing tumor cells and CpG-ODN. We investigated the effects of IL-4 and CpG-ODN on DC maturation by flow cytometry. While the coincubation of DCs with MC38-IL4

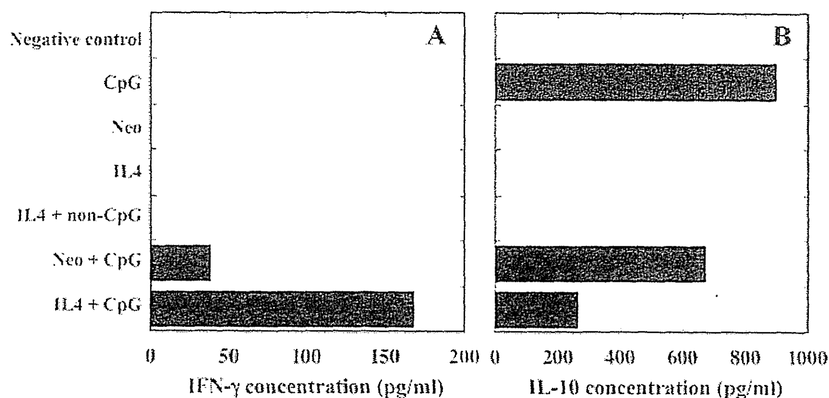

Figure 2. Type-1 T-helper (Th1) cytokine production by naïve splenocytes is enhanced by coincubation with IL-4 and CpG-ODN. Splenocytes from naive mice were stimulated with IL-4-overexpressing or control gene-transduced MC38 cells either alone or in combination with CpG-ODN-1826 or ODN-1911 *in vitro*. After a 24-h incubation, we measured interferon (IFN)-γ and IL-10 concentrations in the culture supernatant by enzyme-linked immunosorbent assay (ELISA).

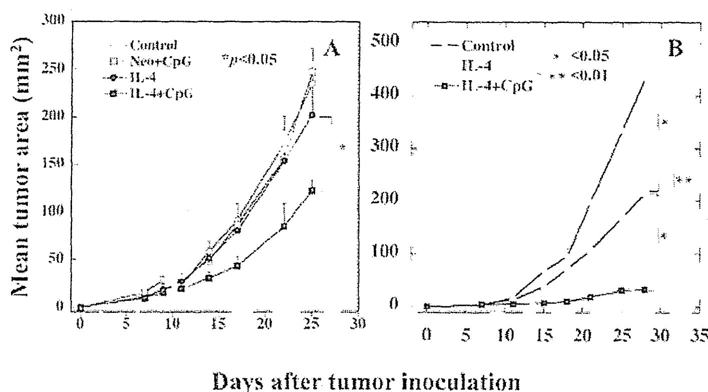

Figure 3. The therapeutic inoculation of IL-4 and CpG-ODN suppresses the growth of parental tumors *in vivo*. B6 mice were injected with MC38-WT cells in the right flank. (A) Seven, 9 and 11 days after the WT inoculation, 3×10^5 cells/mouse MC38-Neo (Control; open circles), MC38-IL4 cells with 30 μg/mouse CpG-ODN-1826 (Neo + CpG; open squares), MC38-IL4 cells with ODN-1911 (IL-4; closed circle), or MC38-IL-4 cells with CpG-ODN-1826 (IL-4 + CpG; closed squares) were inoculated around the established parental tumors, which had reached 4-20 mm^2 in size. (B) Otherwise, tumor-bearing B6 mice were inoculated 5×10^5 cells/mouse MC38-Neo (Control; open circles) or MC38-IL4 cells with either ODN-1911 (IL-4; open squares) or CpG-ODN-1826 (IL-4 + CpG; closed squares) were inoculated in the same manner. Tumor size was measured twice a week. Results are reported as mean tumor area (mm^2) ± SE. Significance at 95% confidence limits is indicated.

enhanced the expression of MHC class I, class II, and costimulatory molecules, only minimal phenotypic changes in DCs were detected after coincubation with CpG-ODN. When we compared DCs that were incubated with IL-4-overexpressing MC38 cells and CpG-ODN-1826 (DC + CpG + IL-4) with the other groups, the expression of H-2K^b, I-A^b, CD80, and CD86 molecules on the DCs was clearly upregulated (Fig. 1). These results suggest that IL-4 and CpG-ODN have synergistic effects on DC maturation.

The combination of IL-4-overexpressing MC38 cells and CpG-ODN promotes the production of Th1-type cytokines by naïve splenocytes in vitro. Since the combination of IL-4 and CpG-ODN enhanced DC maturation, we observed their effects on naïve splenocytes. ELISAs confirmed that naïve splenocytes produced high amounts of IFN-γ after a 24-h coincubation with MC38-IL4 and CpG-ODN-1826 (Fig. 2A), whereas IFN-γ production could not be detected after coincubation with either IL-4 or CpG-ODN alone. While IL-10 production was clearly detected when naïve splenocytes were incubated with CpG-ODN (Fig. 2B), coincubation with MC38-IL4 suppressed the production of IL-10. These results suggest that the combination of IL-4 and CpG-ODN promoted Th1-type immune responses.

Therapeutic inoculation of IL-4-overexpressing MC38 cells and CpG-ODN reduces the outgrowth of established tumors in vivo. Next, we observed whether CpG-ODN had additive antitumor effects on the effects of IL-4 on established tumors *in vivo*. We treated tumor-bearing mice with MC38-IL4 and CpG-ODN-1826 and compared the parental tumor size. As shown in Fig. 3A, the combination therapy of MC38-IL4 (3×10^5 cells/mouse) and CpG-ODN (30 μg/mouse) significantly reduced the outgrowth of the parental tumors (IL-4 vs. IL-4 + CpG, P=0.048) whereas treatment with MC38-Neo + CpG and MC38-IL4 + non-CpG did not show any antitumor effects. Furthermore, the combination of increased cell number of MC38-IL4 (5×10^5 cells/mouse)

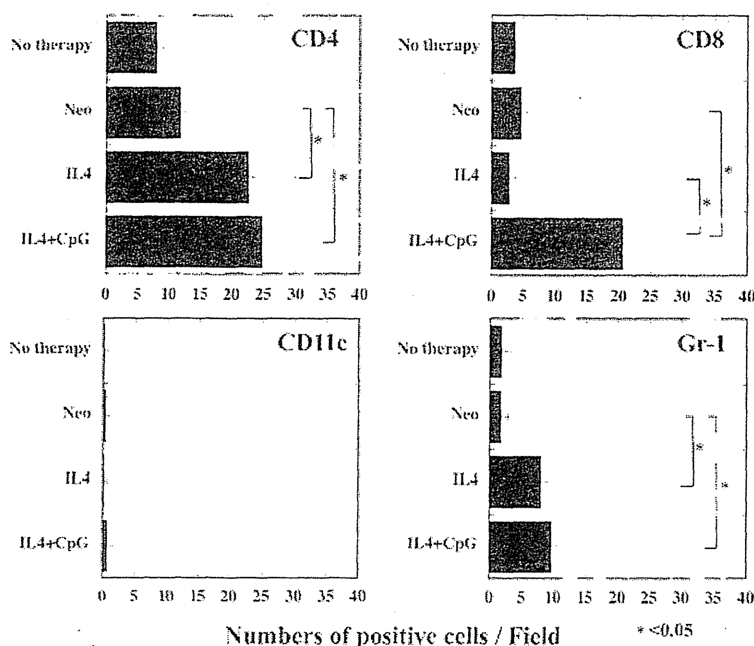

Figure 4. $CD8^+$ cells significantly infiltrate the established wild-type (WT) tumors of mice treated with MC38-IL4 and CpG-ODN. B6 mice were injected with either MC38-Neo or MC38-IL4 cells with or without CpG-ODN-1826 in the area surrounding the established WT tumor 7 days after inoculation of MC38-WT cells. Tumor tissues were harvested 3 days after inoculation of the genetically modified tumor and immediately embedded in optimal cutting temperature compound and frozen for sectioning. Serial 5-μm sections were exposed to anti-Gr-1, anti-CD11c, anti-CD4, or anti-CD8a antibodies. Rat IgG2a was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit. Immunoreactive cells were counted in 6 fields in a light microscope (magnification, x400) in a blinded fashion. Results are shown as mean numbers of positive cells/field ± SD.

and CpG-ODN (30 μg/mouse) was more effective against the established tumors (Fig. 3B; IL-4 vs. IL-4 + CpG, P=0.015) while treatment with MC38-IL4 ($5x10^5$ cells/mouse) only also revealed antitumor activity. Therefore, the combination therapy of IL-4 and CpG-ODN has an additive antitumor effect *in vivo*.

Treatment of IL-4-overexpressing MC38 cells with the combination of CpG-ODN induces the infiltration of CD8-positive cells in established wild-type tumors. We analyzed the mechanisms of the antitumor effects that were induced by the inoculation of MC38-IL4 cells with CpG-ODN-1826. As shown in Fig. 4, WT tumors of mice treated with MC38-IL4 showed significant infiltration with $Gr-1^+$ cells, as previously reported (4). More $CD4^+$ cells infiltrated tumors in mice treated with MC38-IL4 alone, as well as with MC38-IL4 and CpG-ODN, compared with those in mice treated with MC38-Neo (P=0.039 and P=0.026, respectively). While only a few $CD8^+$ cells could be detected in the tumors of mice treated with MC38-IL4 alone, a marked infiltration of $CD8^+$ cells was observed in the tumors of mice treated with MC38-IL4 and CpG-ODN (Fig. 4; MC38-IL4 alone: 2.8±1.9 cells/field vs. MC38-IL4 + CpG-ODN, 20.7±15.3 cells/field, P=0.027). $CD11c^+$ cells were rarely seen in any of the groups. These results suggest that the initial antitumor effects induced by MC38-IL4 and CpG-ODN may be dependent on $CD8^+$ and $CD4^+$ cells.

Potent tumor-specific cytolysis is detected when splenocytes of MC38-immune mice are stimulated by the combination of IL-4-overexpressing tumor cells and CpG-ODN. Because the combination of IL-4 and CpG-ODN seemed to induce potent Th1-type immune responses, we tried to detect the generation of tumor-specific CTL in mice immunized with IL-4 and CpG-ODN. As shown in Fig. 5, effector cells that were stimu-

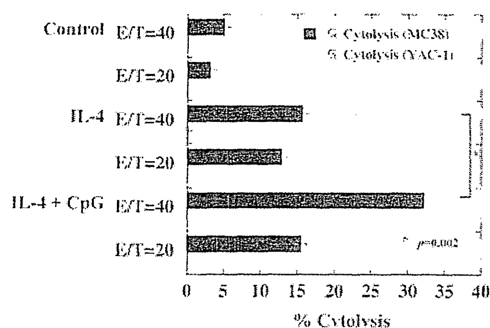

Figure 5. Stimulation with MC38-IL4 and CpG-ODN induces potent tumor-specific cytolysis. Immune mice received injections of both MC38-IL4 cells and control-ODN-1911 (IL-4) or MC38-IL4 cells in combination with CpG-ODN-1826 (IL4 + CpG) on Day 0 and MC38-WT cells on Days 7 and 28. Splenocytes from immunized mice were harvested on Day 42 and stimulated *in vitro* with irradiated MC38-IL4 tumor cells either alone (IL-4) or in combination with CpG-ODN-1826 (IL4 + CpG). Seven days later, responder cells were restimulated with irradiated-IL4 tumor cells either alone (IL-4) or in combination with CpG-ODN-1826 (IL4 + CpG) and supplemented with irradiated syngeneic naive splenocytes as well as recombinant mouse IL-2. Naïve splenocytes that were stimulated with irradiated-IL4 tumor cells and CpG-ODN as described above were also used as control effector cells (Control). A cytolytic assay (4h-^{51}Cr-release assay) against MC38 or YAC-1 cells was performed 6 days after the second stimulation. Results are reported as mean % cytotoxicity ± SD. E:T, effector to target.

lated with IL-4 and CpG-ODN revealed marked cytolysis that was specific for MC38-WT cells (32.2±3.5% for MC38 vs. 3.2±1.1% for YAC-1; at effector to target ratio, E:T=40). Although tumor-specific cytolysis was also detected when the splenocytes of immunized mice that had been inoculated with MC38-IL4 cells and non-CpG-ODN-1911 *in vivo* were stimulated with MC38-IL4 cells *in vitro*, the specific lysis was lower compared to that of the splenocytes of mice treated with IL-4 and CpG-ODN (15.8±2.1% for MC38 vs. 0.3±0.3% for YAC-1; E:T=40, P=0.002 when compared with IL4 + CpG for lysis of MC38). Naïve splenocytes stimulated with IL-4 + CpG-ODN did not show any MC38-specific killing (Fig. 5). The results suggested that immunization with IL-4 and CpG-ODN effectively induce tumor-specific immune response *in vivo*.

Discussion

In this study, we observed that the combination of IL-4 and CpG-ODN enhanced the expression of MHC and costimulatory molecules on the surface of DCs. In addition, the combination promoted IFN-γ production and suppressed IL-10 production in naïve splenocytes *in vitro*, which strongly suggested that the combination of IL-4 and CpG-ODN contributed to T-cell differentiation towards a Th1-type immune response. The combined therapy of IL-4 and CpG effectively suppressed the outgrowth of parental tumors *in vivo* compared to IL-4 monotherapy. The results of both the immunohistochemical and the tumor-specific cytolysis analyses suggested that Th1-type immune responses were strongly induced in mice treated with IL-4 and CpG-ODN.

IL-4, which is a representative Th2-type cytokine, is produced by Th2-type cells. The Th2-type response is thought to suppress the generation of Th1 cells (23), which are usually involved in antitumor immunity. However, it has been reported that IL-4 shows multiple suppressive effects on tumors, and our previous investigation showed that IL-4 gene transduction in MC38 cells did not affect *in vitro* tumor growth, while tumors were not seen in most mice injected with IL-4-overexpressing MC38 cells. This observation implies that IL-4 does not injure MC38 cells directly but reduces their tumorigenicity by inducing host immune responses. Our previous study and other investigations have suggested that IL-4 recruits and activates granulocytes in the microenvironment of parental tumors in order to attack and kill the tumor cells during the primary response. However, how IL-4 recruits or stimulates granulocytes in the tumor microenvironment remains unclear (4,24). Thus, IL-4 induces tumor-specific cellular immune responses, which contribute to long-lasting immunity against the parental tumors.

For further improvement of the antitumor effects of IL-4-based immune therapy, we performed combination therapy, which consisted of IL-4 with CpG-ODN, and this combination has been reported as a potent inducer of immune responses. As previously reported, treatment with CpG-ODN alone did not reduce tumor outgrowth (3). Although CpG-ODN is thought to promote the induction of Th1-type cytokines, CpG-ODN promoted IL-10 production by naïve splenocytes in our system. Reports show that CpG induces Th2-type cytokines in certain conditions support our observation (25,26). MC38-IL4 did not promote IL-10 production, whereas exogenous IL-4 might. We speculate that irradiated tumor cells modified the immune circumstances in the culture. In any case, we suggest that the balance of Th1/Th2 cytokine production is critical to the induction of potent immune responses in the early stages of tumor development and that the great suppression of outgrowth of the parental tumors that was seen in this study occurred because the combination of IL-4 and CpG enhanced IFN-γ production and suppressed IL-10 production. Further investigation is required in order to clarify the mechanisms by which IL-4 reduces the IL-10 production that is induced by CpG.

However, opposite effects of IL-4 on tumors have also been reported. IL-4 itself may affect tumor growth, especially on those that have abundant IL-4 receptors. The levels of expression of the IL-4 receptor correlated with the tumorigenic potential in a murine model (27). Several tumors appeared to be resistant to apoptosis, which is induced by chemotherapeutic agents or CD95 ligation, in an IL-4-dependent manner. IL-4 seemed to modify the immunological functions of effector cells. The cultivation of naïve $CD8^+$ T cells in the presence of IL-4 resulted in poor cytolytic function in the cells by reducing the levels of perforin and granzymes (27,28). Whether an immune response is predominantly Th1 or Th2 may depend on a number of other factors, such as mouse strain, kind of tumor, amount of tumor antigen, and the amount of cytokine (29,30).

It has been reported that the combination of CpG, IL-4, and CD40 ligand effectively induce CTLs by enhancing the expression of immunogenic molecules on B-cell precursor acute lymphoblastic leukemia cells (31). Although MHC class I molecules were slightly upregulated by IL-4 transduction in MC38 cells, changes in other molecules, such as costimulatory and MHC class II molecules, could not be detected in our system (unpublished data). It was thought that IL-4 and CpG-ODN resulted in antitumor effects by inducing Th1-type immune responses, especially that of tumor-specific CTLs, rather than by modulating the immunogenic molecules in the tumor cells. The mechanism by which cytokines and immunomodulators exhibit antitumor effects may also depend on the models used in the experiments.

In order to perform tumor-based gene therapy, there are a number of issues, such as dose determination, site of therapeutic injection, and therapeutic interval, that need to be determined, but the most critical issue is the establishment of the patient's cytokine gene-transduced tumor cells. The development of a new technique that transduces a targeted gene to a patient's cells may be required.

Finally, we found in this study that combination therapy with IL-4 and CpG-ODN had potent antitumor effects on established tumors through the induction of potent Th1-type immune responses in the hosts. Thus, this combination therapy may be a candidate for clinical cancer therapy, but further investigation is needed before clinical trials.

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Original Article

Cyclooxygenase-2 gene promoter polymorphisms affect susceptibility to hepatitis C virus infection and disease progression

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Aim: Because polymorphisms of cyclooxygenase-2 (*COX-2*) and osteopontin (*OPN*) promoter regions and a promoter/enhancer region of forkhead box protein 3 (*FOXP3*) gene are known to affect immune responses, we examined whether these polymorphisms can influence susceptibility to hepatitis C virus (HCV) infection and progression of liver disease.

Methods: Peripheral blood samples were obtained from 104 Japanese patients with chronic HCV infection and 74 healthy Japanese donors. Polymerase chain reaction single-stranded conformational polymorphism analysis of genomic DNA was performed to determine the polymorphisms.

Results: The risk of persistent HCV infection was decreased in subjects with −1195GG genotype of the *COX-2* promoter region. However, in patients with chronic HCV infection, the −1195GG genotype was associated with advanced-stage liver disease. A luciferase reporter assay performed to analyze the effect of single nucleotide polymorphisms (SNP) (−1195A or −1195G) in *COX-2* gene on transcriptional activity using the HepG2, Huh7 and HeLa cell lines indicated that the −1195G genotype showed higher transcriptional activity than the −1195A genotype. SNP of *OPN* and *FOXP3* did not differ between patients with chronic HCV infection and controls. However, the −443TT genotype of the *OPN* promoter region was associated with increased inflammatory activity of the liver.

Conclusion: These results suggest that the −1195GG genotype of the *COX-2* promoter region protects against HCV infection in the Japanese. However, once chronic infection is established, the −443TT genotype of the *OPN* promoter region and the −1195GG genotype of the *COX-2* promoter are thought to promote inflammation and contribute to the progression of liver disease.

Key words: cyclooxygenase-2, forkhead box protein 3, hepatitis C virus, osteopontin, single nucleotide polymorphisms

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection causes chronic hepatitis (CH), liver cirrhosis (LC) and eventually leads to hepatocellular carcinoma (HCC). Immune responses are thought to play important roles in the pathogenesis of viral hepatitis and inflammation is thought to be an important factor in the progression of liver injury.

Cyclooxygenase-1 (COX-1) and COX-2 are enzymes that convert arachidonic acid into prostaglandins and thromboxanes. COX-1 is constitutively expressed in various tissues and plays important roles in homeostasis. In contrast, COX-2 is involved in inflammation, angiogenesis, anti-apoptosis and carcinogenesis.[1–4] COX-2 has been reported to be overexpressed in inflammatory tissues and cancers.[5–8]

The HCV core, NS3 and NS5A proteins are shown to stimulate COX-2 expression.[9,10] Overexpression of COX-2 has been reported in CH, LC and HCC tissues. Furthermore, the COX-2 expression level has been reported to correlate with HCV liver injury activity and

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fibrosis,[11–15] and higher COX-2 expression in the cirrhotic liver has been reported to be a significant independent risk factor for residual liver HCC recurrence after curative surgery for HCC.[15]

Various kinds of transcriptional regulatory factor binding sites and several single nucleotide polymorphisms (SNP) are present in the promoter region of the *COX-2* gene. Some of these SNP influence the risk of esophageal and prostate cancer.[16–19]

Single nucleotide polymorphisms (−616G>T, −443T>C, and −155−>G) are also present in the promoter region of the osteopontin (*OPN*) gene and a promoter/enhancer region of the forkhead box protein 3 (*FOXP3*) ([GT]n) gene. OPN is one of the extracellular matrix proteins that has been identified as an early T-lymphocyte activation antigen (Eta-1) and is produced by activated T cells.[20] It is a key cytokine for the initiation of T-helper cell (Th)1 type immune reaction and promotes tumor metastasis at the carcinoma site.[21] OPN has also been reported to affect various immune responses such as anti-infectious and antitumor immune responses and induce autoimmune disease.[22]

FOXP3 is a transcriptional factor that is mainly expressed in $CD4^+CD25^+$ regulatory T cells (Treg) and it suppresses immune responses. Treg deficiency is one of the mechanisms for initiation and promotion of autoimmune diseases. It has been reported that the frequency of Treg is much higher in people with chronic HCV than that in healthy controls and that Treg suppress HCV-specific immune response.[23–25]

In the present study, we examined the SNP in the promoter regions of the *COX-2* and *OPN* genes and the promoter/enhancer region of the *FOXP3* ([GT]n) gene in patients with HCV infection and studied the relationship between these SNP and susceptibility to HCV infection and the progression of liver disease. We showed that the SNP of the *COX-2* promoter region is involved in susceptibility to HCV infection and progression of liver disease in the Japanese and that the SNP of the *OPN* promoter region affects the inflammatory activities in HCV infection.

METHODS

Patients and blood samples

PERIPHERAL BLOOD SAMPLES were obtained from 104 Japanese patients with chronic HCV infection and 74 healthy Japanese donors. All HCV-infected patients were positive for HCV RNA but negative for hepatitis B surface antigen (HBsAg). Patient characteristics are shown in Table 1. Healthy controls were negative for HCV antibody and HBsAg, and had no autoimmune disease. All patients and controls gave written informed consent according to a protocol approved by the Ethical Committee of Showa University.

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Number	104
Age, years	55.8 (14.2)§
Male : female	57:47
Platelet (10^4/μL)	16.5 (6.6)
AST (IU/L)	57.6 (39.3)
ALT (IU/L)	76.6 (60.1)
γ-GT (IU/L)	72.7 (84.0)
ALP (IU/L)	296.3 (139.7)
Total bilirubin (mg/dL)	0.7 (0.3)
Albumin (g/dL)	4.0 (0.5)
HCV RNA levels† (high : low : ND)‡	92:5:7
HCV genotype (1 : 2 : other and ND)‡	57:28:19

†HCV RNA levels: high ≥100 KIU/mL, low <100 KIU/mL.
‡ND: not determined.
§Mean (SD).
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase.

SNP analysis

Genomic DNA was extracted from peripheral blood using a DNA isolation and purification system (Magtration System 6GC; Precision System Science, Chiba, Japan). DNA polymorphisms in the promoter region of the *COX-2* gene were determined using a fluorescence-based polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis.[26,27] Primer sequences for amplifying the DNA fragment containing the −1195G>A region were 5′-GAGCACTACCCATGATAGATG-3′ (forward) and 5′-TGTTGTACTTTGATCCATGGT-3′ (reverse) and those for the −765G>C region were 5′-ACAGGCTAACTGCTTAGGAC-3′ (forward) and 5′-ACAGCTATGTACACTGAAGG-3′ (reverse). The 5′-end of one of the primers was labeled with cyanine-5 or 6-carboxyfluorescein (6-FAM). The DNA fragments were amplified using ExTaq DNA polymerase (Takara, Shiga, Japan) containing 5% dimethylsulfoxide (DMSO) under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 60°C (−1195G>A region), or 58°C (−765G>C region) for 30 s, and 72°C for 30 s. Nucleotide variations in the DNA fragments were analyzed by the SSCP method

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using an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) or an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide variation of each sample was determined using the wave pattern. The nucleotide sequences of the DNA variations were confirmed by DNA sequence analysis using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer.

The polymorphisms in the promoter region of *OPN* gene were also determined using PCR-SSCP analysis. Primer sequences for amplifying the DNA fragment containing the −616G>T and −443T>C regions were 5′-ACGGTCTGGCTCCTGAAGCA-3′ (forward) and 5′-AGGCTATTGTTCAAGCCTGC-3′ (reverse). The 5′-end of the forward primer was labeled with 6-FAM. The DNA fragment was amplified with Phusion DNA polymerase (Finnzymes, Oy, Finland) containing 0.3% DMSO under the following cycling conditions: 98°C for 30 s, followed by 25 cycles at 98°C for 5 s, 60°C for 10 s, 72°C for 15 s and 72°C for 5 min. The primers used for amplifying the −155–>G region were 5′-ATGCTGAATGCCCATCCCGT-3′ (forward) and 5′-GTCATGAGGTTTTCTGCCAC-3′ (reverse). The 5′-end of the reverse primer was labeled with 6-FAM. The DNA fragment was amplified using ExTaq DNA polymerase containing 5% DMSO under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 30 s. The reaction mixture was applied to an ABI PRISM 3100 Genetic Analyzer.

For analysis of the promoter/enhancer region of the *FOXP3* gene, we amplified the intron zero containing the (GT)n microsatellite polymorphism with the primers 5′-GGTGCTGGACCTCTGCACGT-3′ (forward) and 5′-CCACCTGAGCCACGTGCACA-3′ (reverse). The 5′-end of the forward primer was labeled with 6-FAM. The DNA fragment was amplified with ExTaq DNA polymerase containing 5% DMSO under the following cycling conditions: 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 65°C for 30 s and 72°C for 30 s. Genotyping was performed in a mixture of amplified products and internal size standard by an ABI PRISM 3100 Genetic Analyzer. Reagents and primers were obtained from Sigma Genosys (Hokkaido, Japan) and Exigen (Tokyo, Japan), respectively.

Luciferase assay

To compare the effects of nucleotide variations in the promoter region on COX-2 transcriptional activity, we analyzed promoter activity using a luciferase reporter assay. DNA fragments of the −1630 to the −1 region of the *COX-2* promoter containing the −1195G>A and −765G>C variations were synthesized with the primers 5′-GTAAAACTCGAGCCATGCAATAAATAGGAGTGCC-3′ and (forward) and 5′-GTAAAAAAGCTTGTCGCTAACCGAGAGAACCT-3′ (reverse). DNA fragments were amplified using Phusion DNA polymerase (Finnzymes) containing 5% DMSO under the following cycling conditions: 98°C for 30 s followed by 30 cycles at 98°C for 10 s, 60°C for 30 s, 72°C for 1 min and 72°C for 10 min. The amplified DNA fragments were ligated with the luciferase reporter vector pGL4 (Promega, Madison, WI, USA). The nucleotide sequence of the fragment inserted into each plasmid was confirmed by DNA sequencing. The plasmids were transfected into the HCC cell lines, HepG2 and Huh7 cells, and the human epithelial cervical cancer cell line HeLa using FuGENE HD transfection reagent (Roche, Basel, Switzerland). The pRL-TK plasmid containing the Renilla luciferase gene (Promega) was co-transfected with the pGL4-derived plasmids as an internal standard. At 24 h after transfection, cell extracts were prepared and luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega). Transcriptional activity was determined from the level of firefly luciferase after normalization against Renilla luciferase activity. The transfection process was repeated three times.

Statistical analysis

Odds ratios and 95% confidence intervals for the SNP in HCV infection were calculated by logistic regression and adjusted for sex and age. The relationships between the SNP and platelet counts or serum alanine aminotransferase (ALT) levels were analyzed using the Wilcoxon rank sum or Kruskal–Wallis tests. Activities of the luciferase assay were compared using Student's *t*-test. Statistical analyses were performed using JMP ver. 5 (SAS Institute, Tokyo, Japan). Statistical differences were identified at $P < 0.05$.

RESULTS

−1195GG genotype in the promoter region of the *COX-2* gene was detected less frequently in patients with HCV infection

GENOTYPIC FREQUENCIES OF the SNP in the promoter region of the *COX-2* gene were analyzed in HCV-infected patients and healthy controls. The −1195GG genotype was detected less frequently in

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Table 2 Genotype frequencies of the promoter regions of the *COX-2* and *OPN* genes in patients with chronic hepatitis C virus (HCV) infection and controls

Genotype	Controls $n = 74$ n (%)	HCV $n = 104$ n (%)	OR (95% CI)	*P*-value
COX-2				
−1195G>A				
AA	20 (27.0)	43 (41.3)	Reference	
GA	32 (43.2)	48 (46.2)	0.77 (0.36–1.62)	0.005
GG	22 (29.7)	13 (12.5)	0.23 (0.09–0.59)	
−765G>C				
GG	70 (94.6)	100 (96.2)	Reference	
GC	4 (5.4)	4 (3.8)	0.70 (0.16–3.08)	0.636
CC	0 (0.0)	0 (0.0)	Not calculated	
OPN				
−616G>T				
TT	7 (9.5)	9 (8.7)	Reference	
GT	24 (32.4)	39 (37.5)	1.14 (0.35–3.69)	0.445
GG	43 (58.1)	56 (53.8)	0.74 (0.24–2.28)	
−443T>C				
CC	15 (20.3)	13 (12.5)	Reference	
CT	30 (40.5)	55 (52.9)	2.07 (0.82–5.18)	0.271
TT	29 (39.2)	36 (34.6)	1.47 (0.57–3.80)	
−155–>G				
GG	8 (10.8)	9 (8.7)	Reference	
G–	23 (31.1)	41 (39.4)	1.40 (0.45–4.39)	0.326
– –	43 (58.1)	54 (51.9)	0.83 (0.27–2.49)	

Statistical analysis was performed using multiple logistic analysis and was adjusted for sex and age.
CI, confidence interval; OR, odds ratio.

HCV-infected patients than in healthy controls (Table 2). No significant difference was detected in the frequency of SNP of the −765G>C SNP between patients and controls. The frequencies of the −1195G>A and −765G>C SNP in the healthy controls were similar to those obtained from a large Japanese population study.[28] These results suggest that the −1195GG genotype is protective against HCV infection.

−1195GG genotype of the *COX-2* promoter region contributes to liver injury progression in patients with chronic HCV infection

We investigated the relationship between the SNP and platelet counts in patients with chronic HCV infection to evaluate whether the SNP of the *COX-2* promoter region was involved in the progression of liver disease because platelet counts have been reported to reflect the stages of chronic HCV infection and liver fibrosis.[29,30] Patients with the −1195GG genotype had significantly lower platelet counts than those with the −1195AA or AG genotype (median [range] $12.8 \times 10^4/\mu L$ [6.0–$25.9 \times 10^4/\mu L$] vs $16.9 \times 10^4/\mu L$ [5.2–$34.0 \times 10^4/\mu L$]) (Fig. 1). The results suggest that, once chronic HCV infection is established, the −1195GG genotype contributes to the progression of liver disease.

COX-2 promoter region containing the −1195G genotype showed significantly higher transcriptional activity than that containing the −1195A genotype in cell lines *in vitro*

To analyze the effect of SNP on transcriptional activity, we constructed a plasmid containing the −1195A and −765G SNP or the −1195G and −765G SNP. The plasmids were co-transfected with pRL-TK into the HCC cell lines HepG2 and Huh7 and the epithelial cervical cancer cell line HeLa. At 24 h after transfection, we

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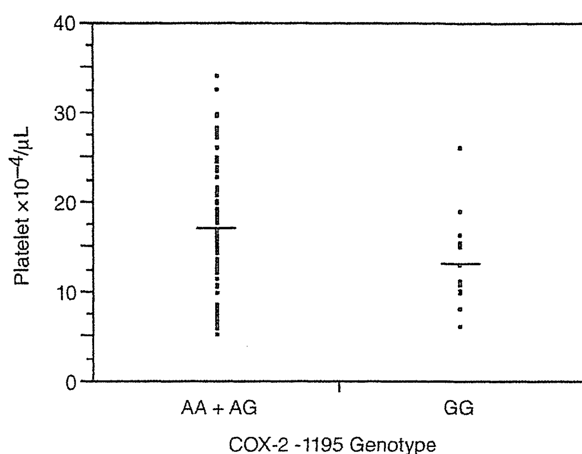

Figure 1 The −1195G>A genotype of the promoter region of the *COX-2* gene and platelet counts in patients with chronic hepatitis C virus infection. Platelet counts were significantly lower in patients with the −1195GG genotype of the promoter region of the *COX-2* gene than those with the −1195AA or AG ($P = 0.04$). The horizontal lines on the plots indicate the means.

determined the transcriptional activity by dual luciferase reporter analysis. When the activity of the promoter region containing −1195A was defined as 100%, the relative activity (mean ± standard deviation) of that containing −1195G was 169 ± 42% in HepG2 cells, 162 ± 38% in Huh7 cells and 154 ± 34% in HeLa cells (Fig. 2). The promoter region containing −1195G showed significantly higher transcriptional activity than that containing −1195A in all three cell lines. The results suggest that the COX-2 promoter region containing the −1195G allele increases transcriptional activity in liver cells and enhances COX-2 expression. Because the −1195GG genotype was more frequently observed in patients with low platelet counts, the high levels of COX-2 expression would be involved in the progression of liver injury.

Association observed between the −443T>C genotype of the OPN promoter region and ALT levels of patients with chronic HCV infection

We also examined the SNP of the promoter region of OPN (−616G>T, −443T>C and −155−>G) and found no significant differences between chronic HCV patients and controls (Table 2). However, patients with the −443TT genotype had significantly higher serum ALT levels than those with the −443CC or CT genotype (median [range] 76 IU/L [17–319 IU/L] vs 46 IU/L [9–266 IU/L]) (Fig. 3).

No relationship between microsatellite polymorphisms of the promoter/enhancer region of the *FOXP3* (GT)n gene and HCV infection.

We separately analyzed the polymorphisms in women and men because the *FOXP3* gene is located on chromosome Xp11.23. We determined the frequency of the $(GT)_{15}$ polymorphism because it has been reported that the major $(GT)_{15}$ dinucleotide repeat has stronger enhancer activity than that of the $(GT)_{16}$ repeat by a luciferase reporter assay using HeLa, COS-7 and Jurkat T cells.[31] However, we detected no differences in the polymorphisms between HCV positive patients and controls (Table 3).

DISCUSSION

IN THE PRESENT study, we found that the frequency of the −1195G>A genotype of *COX-2* was significantly different between patients with chronic HCV infection and healthy controls in the Japanese. We also found that the transcriptional activity of the *COX-2*

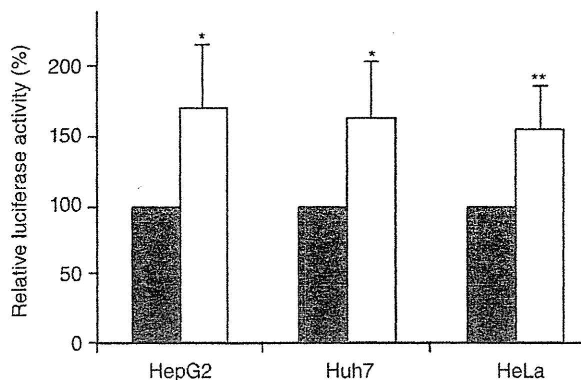

Figure 2 Comparison of the transcriptional activity of the *COX-2* promoter region containing the −1195A and −765G (closed bar) and that containing the −1195G and −765G (open bar) in HepG2, Huh7 and HeLa cells using a luciferase reporter assay as described in Methods. The average relative luciferase activity is shown from three independent transfection experiments, and each was performed in triplicate. The activity of the COX-2 promoter region containing the −1195A was defined as 100%. The vertical lines above bars indicate the standard deviations $*P < 0.05$, $**P = 0.05$.

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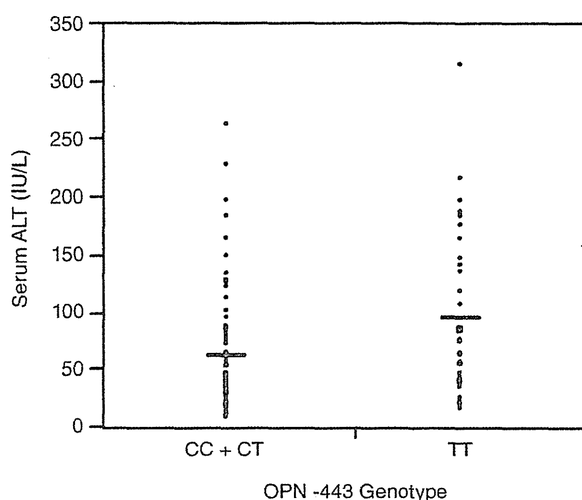

Figure 3 Relationship between the –443T>C genotype of the promoter region of the *OPN* gene and alanine aminotransferase (ALT) levels in patients with chronic hepatitis C virus infection. ALT levels were significantly higher in patients with the *OPN* promoter region containing –443TT genotype than those with the –443CC or CT genotype ($P = 0.01$). The horizontal lines on the plots indicate the means.

gene with the −1195G variant is significantly higher than that with the −1195A variant. We confirmed this by using three cell lines. However, one report states that the transcriptional activity of the *COX-2* gene with the −1195A variant is higher than that with the −1195G variant in HeLa cells,[16] which were also used in our experiment. These contrary results may be due to the different lengths of the DNA fragments used in the experiments. On the basis of our results, we assume that the −1195G variant has much more activity than the −1195A variant in hepatocytes. The −1195GG genotype was observed less frequently in patients with chronic HCV infection compared with HCV non-infected controls and was observed more frequently in patients with low platelet counts. These results suggest that the −1195G allele may resist HCV infection by inducing strong COX-2 expression. It has been reported that, indeed, prostaglandins affect both promotion and inhibition of virus replication.[32–35] However, once persistent HCV infection is established in hepatocytes, the −1195GG genotype promotes liver inflammation by inducing strong COX-2 expression and progressing liver injury. Genetic variation of this site may not only alter transcriptional gene activity and affect HCV infection susceptibility but also enhance HCV-induced liver disease progression.

Interferon (IFN) is used to eradicate HCV infection and reduce HCV-related liver damage. IFN treatment has been reported to reduce COX-2 expression in the liver in chronic HCV.[36] The promoter region of *COX-2* contains several transcription binding sites (C/EBP, AP2, SP1, NF-κB, CRE, Ets-1, PEA-3 and GATA-1).[37,38] The HCV NS3 protein enhances *COX-2* gene promoter activity, *COX-2* mRNA expression, COX-2 protein production and prostaglandin E2 release in HepG2 cells, all of which are regulated by NF-κB and multiple signaling components including JNK, ERK and PKD2.[9] The HCV core and NS5A proteins upregulate *COX-2* gene expression in hepatocyte-derived cells.[10] The genetic variant −1195G in the *COX-2* promoter region enhanced the promoter activity in our experiments. It is thought that the −1195G allele and HCV have an additive effect on enhancement of COX-2 expression, which affects transcription binding sites.

Because there is a close relationship between HCV infection and COX-2 expression, reduction of COX-2 expression may help control HCV-induced chronic liver injury. Unlike COX-1, COX-2 expression is undetectable in most normal tissues. In HCV-induced liver injury, inhibition of COX-2 expression may have two therapeutic potentials: improvement of hepatic inflammation and suppression of carcinogenesis. It has been reported that COX-2 inhibitors can promote apoptosis and sup-

Table 3 Genotype frequencies of the promoter/enhancer region of the *FOXP3* gene in patients with chronic hepatitis C virus (HCV) infection and controls

	Women			Men		
	n	Genotype		*n*	Genotype	
		$(GT)_{15}/(GT)_{15}$	others		$(GT)_{15}$	others
Control	32	7 (21.9)†	25 (78.1)	42	15 (35.7)	27 (64.3)
HCV	49	11 (22.4)	38 (77.6)	55	21 (38.2)	34 (61.8)

†Number (%).

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press growth of a human hepatoma cell line.[39,40] Thus, COX-2 may be a target for preventing progression to cirrhosis, development of HCC, and recurrence of HCC after surgical or local therapy. Indeed, we showed that the −1195GG genotype was observed more frequently in patients with low platelet counts. This suggests that the −1195GG genotype would contribute to progression of liver injury. COX-2 inhibitors may reduce liver damage. This would be an attractive approach for patients who were not able to achieve sustained virological responses by IFN therapy. However, COX-2 inhibitors have several serious side-effects such as renal, gastrointestinal and cardiovascular problems. New COX-2 inhibitors without serious side-effects would be needed to treat patients with chronic HCV infection.

Although there was no difference in SNP of the promoter region of the *OPN* gene between healthy controls and patients with chronic HCV infection, the −443TT genotype was associated with increased levels of ALT. The frequency of the −443TT genotype has been reported to be higher in patients with high ALT levels.[41] Thus, an SNP of the *OPN* promoter region of −443T>C may affect hepatitis activity. No difference was observed in SNP of the promoter/enhancer region of the *FOXP3* gene between patients with chronic HCV infection and healthy controls.

In conclusion, our results suggest that the −1195GG genotype of the *COX-2* promoter region is protective against HCV infection in the Japanese. However, once chronic infection is established, the −443TT genotype of the *OPN* promoter region and the −1195GG genotype of the *COX-2* promoter are thought to promote inflammation and contribute to the progression of liver disease.

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Review Article

Immune Response of Cytotoxic T Lymphocytes and Possibility of Vaccine Development for Hepatitis C Virus Infection

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Immune responses of cytotoxic T lymphocytes (CTLs) are implicated in viral eradication and the pathogenesis of hepatitis C. Weak CTL response against hepatitis C virus (HCV) may lead to a persistent infection. HCV infection impairs the function of HCV-specific CTLs; HCV proteins are thought to actively suppress host immune responses, including CTLs. Induction of a strong HCV-specific CTL response in HCV-infected patients can facilitate complete HCV clearance. Thus, the development of a vaccine that can induce potent CTL response against HCV is strongly expected. We investigated HCV-specific CTL responses by enzyme-linked immuno-spot assay and/or synthetic peptides and identified over 40 novel CTL epitopes in the HCV protein. Our findings may contribute to the development of the HCV vaccine. In this paper, we describe the CTL responses in HCV infection and the attempts at vaccine development based on recent scientific articles.

1. Introduction

Hepatitis C virus (HCV) was first identified in 1989 [1]. The HCV is a member of the flavivirus family and is a type of positive-strand RNA virus. The discovery of HCV contributed to the diagnosis of hepatitis C; further, HCV has been implicated in many chronic non-A and non-B hepatitis infections. This virus spreads through needles used for vaccination or drug administration, and about 180 million people in the world are presumed to be infected with HCV. It has been clarified that HCV infection often persists, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

Cytotoxic T lymphocyte (CTL) plays a part in viral eradication [2]. These cells have been also implicated in the immunopathogenesis of viral infection [3], because HCV, by itself, does not produce cytopathic effects in hepatocytes directly. It has been thought that hepatitis is caused by the destruction of HCV-infected hepatocytes by immune cells such as natural killer (NK) cells and CTLs. Thus, the investigation of the roles of CTL in immunopathogenesis of HCV would contribute to the development of a new treatment strategy for HCV-induced hepatitis.

Interferon (IFN) therapy alone or with ribavirin and polymerase/protease inhibitor combination therapy has shown positive outcomes in more than 80% of patients with acute HCV infection and 50% of patients with chronic HCV infection. However, IFN causes severe adverse effects including flu-like symptoms, pancytopenia, hyperglycemia, depression, lung fibrosis, and cerebral bleeding. Therefore, there is an urgent need to establish an alternative therapy, which can afford a high rate of sustained virological response and performed with few adverse effects. Immunotherapy with HCV vaccine is one of the candidates of such therapies.

In this review, we have summarized the findings of recent investigations on CTL responses against HCV and the trials for the development of HCV vaccine.

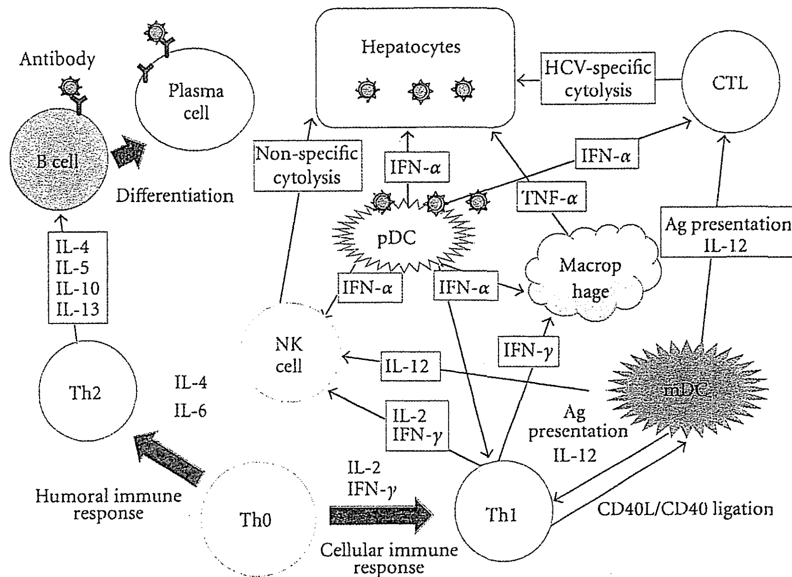

Figure 1: Cellular and humoral immune responses in HCV infection. Plasmacytoid dendritic cells (pDC) recognize HCV infection and produce IFN-α, which activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTLs). Activated NK cells destroy the HCV-infected hepatocytes in a nonspecific manner, whereas CTLs destroy the infected hepatocytes in an antigen-specific manner. Myeloid dendritic cells (mDC), which recognize dead hepatocytes, secrete IL-12, promoting the activation of NK cells, Th1 cells, and CTLs. Activated Th1 cells, in turn, promote DC maturation by interacting with the CD40/CD40 ligand. Macrophages stimulated by type 1 helper T (Th1) cells produce TNF-α, which accelerates local inflammation. In humoral immune responses, Th2 cells activate B cells. Plasma cells differentiated from B-cells secrete immunoglobulins to neutralize the circulating HCV. Abbreviated terms: CTL, cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th1 cell, type 1 helper T cell; Th2 cell, type 2 helper T cell; NK cell, natural killer cell; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

2. CTL Responses in HCV Infection

2.1. Innate Immune Responses in HCV Infection. HCV infection induces cellular and humoral immune responses (Figure 1). Similar to other viral infections, nonspecific immune responses are induced in the early stages of HCV infection for the eradication of HCV. Type I IFNs produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DCs) suppress viral replication by inducing enzymes such as 2′–5′ oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes [4]. The plasmacytoid DC recognizes HCV infection through toll-like receptor (TLR)-7, which interacts with single-stranded RNA [5]. The TLR-signaling upregulates PDC-TREM molecules on the cell surface, and PDC-TREM-dependent signal induces further production of IFN-α [6]. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysome of viral mRNA [4]. Moreover, type I IFNs activate innate immunity components such as natural killer (NK) cells [7]. The local inflammation further activates natural killer T-cells (NKT cells) and macrophages (Kupffer cells), thereby inducing the production of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α. Hepatitis is thought to be initiated in this manner, and specific immune responses are generated if innate immune responses fail to eradicate HCV.

2.2. HCV-Specific Immune Responses and Immunopathogenesis of HCV-Specific CTLs. The process of HCV-specific CTL induction and the destruction of HCV-infected hepatocytes by CTLs are shown in Figure 2. The destruction of HCV-infected hepatocytes releases HCV fragments; these fragments are taken up by myeloid DCs, consequently activating the DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on human leukocyte antigen (HLA) class II molecules. Then, they enhance expression of costimulatory molecules (CD80, CD86) that interact with and activate antigen-specific helper T (Th) cells [8]. In turn, the activated Th cells promote the maturation of DCs by the expression of CD40 ligand and TNF-α. Subsequently, mature DCs stimulate specific CTLs by antigen presentation on HLA class I molecule and enhance the expression of costimulatory molecules [8]. Cytokines such as IL-2 and IL-12 produced by Th1 cells and DCs further promote CTL activation. These CTLs infiltrate the liver and recognize HCV antigens presented on the surface of HCV-infected hepatocytes together with HLA class I molecules. Then, the effector CTLs release perforin, granzyme, and TNF-α, or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes [9, 10]. In the previous study, we demonstrated that Fas ligand and TNF-α can also destroy noninfected hepatocytes in the vicinity of the HCV-infected cells [11].

When appropriate CTL responses are induced in hosts, HCV eradication is achieved. However, HCV-specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection. On the

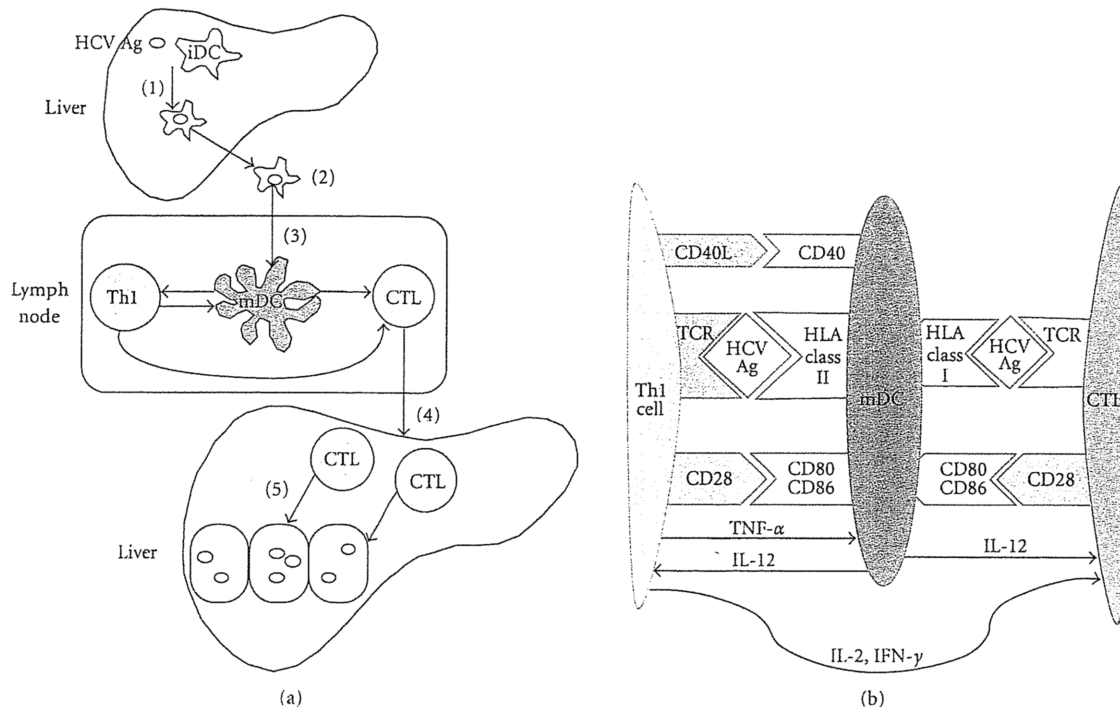

FIGURE 2: Destruction of HCV-infected hepatocytes by CTLs. (1) Immature myeloid dendritic cells (iDC) take up hepatitis C virus antigens (HCV Ag) in the liver. (2) The DCs move to a draining lymph node. (3) Matured DCs activate naïve helper T (Th) cells efficiently through stimulation with HLA class II, costimulatory molecules (CD80 and CD86), and cytokines such as IL-12. The stimulated Th cells, in turn, activate DCs by expressing CD40 ligand and secreting TNF-α. IL-12 produced by myeloid DCs differentiates these stimulated Th cells towards Th1 cells. Naïve cytotoxic T lymphocytes (CTLs) recognize the HCV Ag presented on the DCs. IL-2 and IFN-γ secreted by activated Th1 cells induce the activation and proliferation of the HCV-specific CTLs. (4) The stimulated HCV-specific CTLs leave the lymph nodes and move toward the liver. (5) They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and try to eradicate HCV by killing the infected hepatocytes. Abbreviated terms: Th1 cell, type 1 helper T cell; mDC, myeloid dendritic cells; CTL, Cytotoxic T lymphocyte; CD, cluster of differentiation; CD40L, CD40 ligand; TCR, T-cell receptor; HLA, human leukocyte antigen; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

other hand, markedly potent immune responses would lead to severe hepatitis and fulminant hepatitis as proven in a hepatitis B virus (HBV) model [12], although this is a rare event in HCV infection.

We evaluated the relation between HCV-specific CTL responses and the clinical course of acute HCV infection and found that HCV eradication cannot be predicted on the basis of a strong CD8$^+$ T-cell response [13]. However, Lauer et al. reported that potent and broad CTL responses against HCV peptides were observed in patients with resolved infection but not in those with persistent infection [14]. Another report indicated that patients with complete resolution of HCV infection exhibited broader CTL responses with higher functional avidity and wider cross-recognition ability than patients with persistent HCV infection [15]. The opposite observations can be attributed to the differences in the monitoring methods of the CTL responses. Race and HCV genotype might also affect the contradiction of the results. Further investigation is needed to clarify this issue.

We analyzed the immune response of chronic HCV patients by studying their HLA-B44-restricted CTLs that recognized the HCV core amino acid residues 88–96; the CTL response and viral load were found to be inversely correlated [16]. The findings of this study suggested that HCV-specific CTLs may inhibit HCV replication. Otherwise, as many reports have suggested that HCV protein impairs the CTL responses by several mechanisms (see Section 3), HCV infection with a high titer of HCV RNA may suppress the HCV-specific CTLs by an excess of HCV antigens. No relation between other CTL responses recognizing other HCV epitopes and the HCV status was found in the study. From the data, it was supposed that the HLA-B44-restricted CTLs recognizing HCV core amino acid residues 88–96 were immunodominant.

Hence, there is a need to investigate HCV-specific CTL responses and clarify some issues. First, HCV exists as quasispecies in hosts and it has a high replicative ability and low fidelity RNA polymerase [17]. Thus, many HCVs with mutations in different amino acid sequences in the epitopes may be present in the host. Other issue is that most HCV-specific CTLs may infiltrate and compartmentalize in the host liver where inflammation occurs, and thus, only a few circulating HCV-specific CTLs can be detected. Although it is very crucial to investigate liver-infiltrating CTLs, the difficulty associated with obtaining liver specimen limits such study.

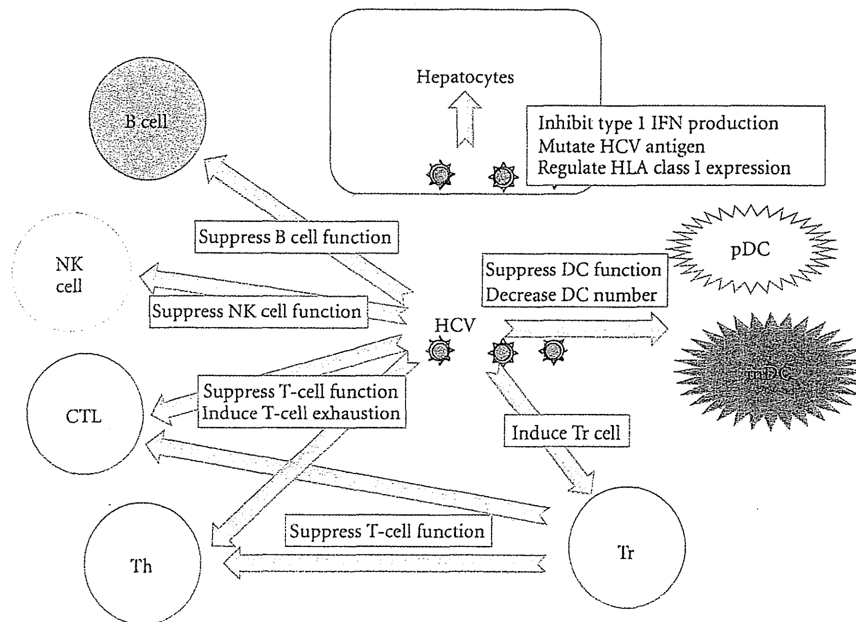

FIGURE 3: Immune suppressive mechanisms in HCV infection. HCV mutates its amino acid sequence to escape from immune surveillance, inhibits type 1 IFN production, and suppress NK cell function, T-cell function, and DC function. In addition, HCV induces Tr cells, which suppress T-cell function. Abbreviated terms: CTL, Cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th cell, helper T cell; NK cell, natural killer cell; IFN, interferon.

3. Immunosuppression in HCV Infection

3.1. Escape from Immune Surveillance of Cellular Immune Responses. It was reported that amino acid mutations have been detected in the immunodominant regions of HCV in all patients with acute HCV infection, and mutations by which HCV escapes from CTL surveillance have been observed only in patients with viral persistence [18]. Hughes et al. investigated the variable intensity of purifying selection on CTL epitopes, and reported that the purifying selection of CTL epitopes on nonenvelop proteins was strong, particularly when the epitope was matched [19]. Since a variety of CTLs are induced in the early stage of HCV infection, a single amino acid mutation within a CTL epitope does not appear to contribute to persistent infection. It is supposed that escape mutation is a result rather than a cause of persistent HCV infection.

3.2. Impaired Function of CTL in HCV Infection. HCV inhibits cellular immune responses in the host by several ways; immune suppressive mechanisms in HCV infection are summarized in Figure 3.

In our study, the stimulation of peripheral blood lymphocytes of HCV-infected patients with synthetic peptides corresponding to CTL epitopes revealed that patients who were infected with HCV within the past 3 years exhibited CTL responses, while those infected with HCV more than 10 years ago did not exhibit this response. There are some reasons why HCV persistence is so common although a variety of HCV-specific $CD8^+$ T-cells can be detected in the liver and peripheral blood. The impaired function of HCV-specific CTLs as effector cells is due to the reduced expression of CD3 ζ chain [20], defective IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity [21]. Incomplete differentiation of the memory CTLs to effector cells in patients with acute HCV infection may be due to IL-2 deficiency during T-cell activation [22]. Programmed cell death 1 (PD-1) receptor, the ligation of which inhibits the function of effector T-cells, is upregulated on exhausted $CD8^+$ cells in patients with acute and chronic hepatitis C [23–25]. Another inhibitory receptor, namely, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has also been reported to be upregulated on PD-1^+ T-cells in the liver of HCV patients. The blockade of both these molecules is critical for the restoration of the function of HCV-specific effector cells [26].

Accumulated data have suggested that HCV itself actively suppresses host immune responses. Although spontaneous liver disease did not occur in mice expressing liver-targeted HCV NS5A transgene, both innate and adaptive immune responses were impaired [27]. HCV core protein inhibits IL-2 and IL-2 receptor α gene transcription [28], T-cell activation and proliferation, and IFN-γ production by T cells [29, 30]. HCV NS4A/B protein blocks the expression of HLA class I molecules [31].

Impaired function of DCs, which play the crucial role of antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. It has been reported that the HCV core, E1, and NS3 proteins inhibit DC maturation [32, 33]. HCV is thought to infect DCs through the binding of HCV E2 protein and thereby suppress

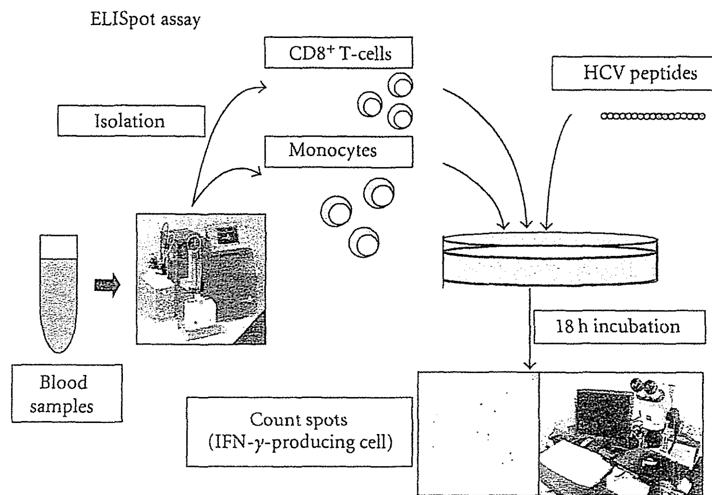

FIGURE 4: The procedure of enzyme-linked immuno-spot (ELISpot) assay. To detect CTL responses against HCV, we performed IFN-γ-based ELISpot assay. $CD8^+$ cells and monocytes were separated from peripheral blood samples by magnetic beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and used as effector cells and antigen-presenting cells, respectively. These cells with synthetic HCV peptides were incubated for 18 h at 37°C in 5% CO_2 atmosphere. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted.

DC function [34, 35]. In addition, long-term ethanol consumption impairs CTL responses to HCV protein and subsequently alters DC function [36].

Regulatory T- (Tr) cells are also involved in HCV persistence. It has been shown that Tr cells ($CD4^+$ $CD25^+$ T cells) directly suppress T-cell function in chronic hepatitis C patients [37]. Forkhead box P3 (FOXP3)-positive Tr cells and IL-10 producing HCV-specific Tr cells infiltrate the liver of chronic HCV patients, and IL-10 mediates immune suppression in these patients [38, 39]. HCV core-specific Tr cells can be induced from the peripheral blood of patients with chronic hepatitis C [40].

4. Immunotherapy for Hepatitis C

4.1. IFN Therapy and Immune Response. Currently, chronic HCV infection can be resolved only with IFN-α-based therapy. IFN-α has been reported to have biologic effects on the immune system [41]. IFN-α upregulates HLA class I molecules on the cell surface. This cytokine appears to favor the proliferation of type 1 Th cells and activate CTLs. Ribavirin, which is used in combination with IFN-α, exerts an antiviral effect that drives the Th2 response towards a Th1 response [42]. During the primary immune response, IFN-α promotes both clonal expansion and survival of antigen-specific CTLs in vivo [43]. We also demonstrated that IFN-α prevents activation-induced cell death of CTLs [44]. A low dose of IFN-α augments cellular immune response, whereas a high dose suppresses CTL response [45]. Recently, it has been reported that although IFN-α upregulates MHC class I expression on hepatocytes, it reduces their sensitivity to CTL cytotoxicity, which may be due to the enhancement of granzyme-B inhibitor-proteinase inhibitor 9 (PI-9) expression [46]. Although it has been reported that intrahepatic and peripheral HCV-specific CTL activity was detected more often in patients with a sustained response to IFN therapy than in patients who relapsed or did not respond to the treatment [47], further study is needed to clarify the effect of IFN therapy on host immune responses in vivo.

4.2. Identification of Novel Epitopes Recognized by HCV-Specific CTLs. As described above, we first identified an HLA B44-restricted CTL epitope [48, 49]. Then, we tried to identify more novel CTL epitopes in the HCV polyprotein, and performed IFN-γ-based enzyme-linked immuno-spot (ELISpot) assay [50, 51]. The procedure of this assay is presented in Figure 4. We synthesized 297 20-mer peptides overlapping by 10 residues and spanning the entire HCV sequence based on the amino acid sequence of HCV [13]. After separation with magnetic beads, we used $CD8^+$ T-cells as effector cells and monocytes as antigen-presenting cells. After the $CD8^+$ T-cells were incubated with the monocytes and the synthetic HCV peptides for 18 hours, IFN-γ-producing cells were counted. This procedure enabled to minimize the IFN-γ production for nonspecific response. Then, we identified more than 20 CTL epitopes in the HCV protein by using the synthetic peptides (Table 1). Furthermore, our group has identified several epitopes of HCV-specific CTLs using synthetic peptides and recombinant vaccinia viruses [52].

The HLA-24 allele of HLA class I is more common among the Japanese population. Thus, CTL induction by synthetic peptides based on HLA-A24 binding motifs has been investigated mainly in Japan [53]. HCV NS5A 2132–2142 peptide corresponding to the HLA-A24 binding motif has been reported to be able to induce both cellular and humoral immune responses in most HCV-positive patients

Table 1: CTL epitopes identified by using different procedures.

(a) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt1	A*0207,2601 B*3501,4601 CW*0102,0303	NS3	1527–1546	WYELTPAETTVRLRAYLNTP	B*3501? A*2601?
		NS5B	2591–2605	KMALYDVVSTLPQAV	A*0207?
Pt2	A*2402,3303 B*4403,5401 CW*0803,1403	E1	332–351	LVVSQLLRIPQAVVDMVAGA	B*5401?
		NS3	1638–1656	THPITKFVMACMSADLEVV	B*5401?
		NS5B	2591–2605	KMALYDVVSTLPQAV	n.d.
Pt3	A*2602,3101 B*5101,5102 Cw1402,1502	NS3	1373–1380	IPFYGKAI	B*5101? B*5102?
Pt4	A*2402 B*0702,5201 Cw*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt5	A*1101,3101 B*6701,5101 Cw*0702,1401	NS5A	2290–2298	RPDYNPPLL	B*6701? B*5101?
Pt6	A*2402,2601 B*4002 Cw*0304	NS2	957–964	RDWAHAGL	B37
		NS5A	2122–2130	FTELDGVRL	n.d.
Pt7	A*2402;3303 B*0702,3501 Cw*0303,0702	Core	91–110	LGWAGWLLSPRGSRPSWGPT	A*3303? B*3501?
Pt8	A*2402 B*4801,5201 Cw*0803,1202	NS3	1643–1656	KFVMACMSADLEVV	n.d.
Pt9	A*2402 B*5201 Cw*1202	NS4	1760–1768	FWAKHMWNF	A*2402
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt10	A*0201,0301 B*4402,4601 Cw*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt11	A*1101,2601 B*1501,5201 Cw*0401,1202	NS4	1858–1867	GVAGALVAFK	A*1101?
Pt12	A*2402 B*3501,4002 Cw*0303,0304	NS3	1618–1626	LHGPTPLLY	A*2402?

(b) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-A24 [51]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt13	A*2402,1101 B*3902,5201 Cw*0702,1202	NS3	1375–1385	FYGKAIPIEAI	n.d.
Pt14	A*2402,2601 B*4006,5401 Cw*0801,0803	E1	284–293	VFLVSQLFTF	n.d.
		E2	790–798	LYGVWPLLL	Cw*0801
		NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5A	1990–1999	DFKTWLQSKL	n.d.
		NS5A	2280–2288	KFPPALPIW	A*2402
Pt15	A*2402,2601 B*3501,4002 Cw*0303,0304	NS2	910–919	PYFVRAQGLI	Cw*0303, 0304
		NS2	947–956	TYVYDHLTPL	B*4002
		NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304
Pt16	A*0206,2402 B*5201,5901 Cw*0102,1202	NS3	1443–1451	GFTGDFDSV	A*0206
Pt17	A*2402,3101 B*4801,5101 Cw*0304,0801	E2	790–798	LYGVWPLLL	Cw*0801
Pt18	A*2601,3101 B*3501,5101 Cw*0303,1402	NS5B	2456–2466	VYSTTSRSASL	n.d.

(c) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence [13]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt19	A*2602,3101 B*5101,5102 C*1402,1502	NS3	1373–1380	IPFYGKAI	n.d.
Pt20	A*0402 B*0702,5201 C*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt21	A*1101,3101 B*6701,5101 C*0702,1402	NS5A	2290–2298	RPDYNPPLL	n.d.
Pt22	A*2402 B*5201 C*1202	NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt23	A*0201,0301 B*4402,4601 C*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt24	A*2402,4801 B*5201 C*0803,1202	NS3	1637–1656	LTHPITKFVMACMSADLEVV	n.d.

(d) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV core sequence

Region	Amino acid residues	Sequence	HLA restriction	Reference
core	88–96	NEG(L,M,C)GWAGW	B*4403	[49]
core	28–36	GQIVGGVYL	B60	[50]

(e) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-B*4403

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS5a	2095–2103	AEVTQHGSY	B*4403	[16]

(f) CTL epitopes identified by comprehensive CTL induction from PBMC of HCV patients

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS3	1373–1380	IPFYGKAI	B*5603	[52]

with HLA-A24 [54]. Three novel vaccine candidate peptides capable of CTL induction and antibody production have also been identified [55]. In the study, the HCV core 30–39 peptide was shown to induce peptide-specific CTLs from peripheral blood mononuclear cells (PBMCs) of patients with HLA-A11, -A31, or -A33.

Yerly et al. [56] developed a novel "epitome" approach and analyzed its in vitro performance. This approach compresses the common immune targets of HCV-specific cellular immune response into a short immunogen sequence and may be applied to induce cellular immune responses against highly variable antigens.

The most important concern in peptide vaccine development is the selection of peptides from among the CTL epitopes because some peptides may rather induce tolerance of effector cells [57] or Tr cells, which will result in immune suppression. Hence, it is necessary to develop tailor-made therapy using appropriate peptides according to the HLA haplotypes of the patients.

4.3. Trials for the Development of HCV Vaccine. Many attempts for inducing immune responses against HCV by vaccination have been performed using animal models. Splenocytes isolated from mice pretreated with Fms-like tyrosine kinase receptor 3 ligand exhibited NS5-specific cellular immune responses after vaccination with DCs containing magnetic beads coated with HCV NS5, lipopolysaccharide, and anti-CD40 antibody [58, 59]. It has been reported that the adoptive transfer of HCV NS3 protein-pulsed mature DCs could effectively promote potent HCV-specific protective immune responses in a mouse model [60]. From the data, DC-based therapy appears to be one of the candidates for immune therapy against HCV infection.

Since HCV envelope glycoproteins are heavily glycosylated, such modification would affect immune responses in hosts. The engineering of *N*-glycosylation of HCV E2 protein enhances HCV-specific cellular immune responses [61], whereas the deletion of *N*-glycosylation sites of HCV E1 protein augmented HCV-specific cellular and humoral immune responses [62].

Gene therapy has been tried to elicit strong immune responses in vivo. It has been reported that vector-based minigene encompassing 4 domains of HCV NS3, NS4, and NS5B proteins effectively induced CTL induction in HLA-A2 transgenic mice [63]. Using replication-incompetent adenoviruses expressing HCV core and NS3 proteins, HCV-specific CTLs could be induced from PBMCs of HCV-infected patients [64]. Administration of recombinant yeast cells producing HCV NS3-core fusion protein, namely, GI-5005, induced potent antigen-specific proliferative and CTL responses in mice [65]. As described above, gene therapy would be a candidate for HCV vaccine. However, a careful survey for adverse effects induced by the therapy must be performed before clinical application.

Adjuvants may help the induction of HCV-specific CTLs, and it is important to investigate what adjuvant we should use for HCV vaccination. Protein immunization using CpG and montanide ISA 720 have been reported to enhance HCV-specific Th-1 type immune responses [66]. Cytokines such as granulocyte-monocyte colony stimulating factor and IL-23 have been also used for argument of immune responses induced by HCV core vaccination [67]. In a mouse model, HBV precore protein enhanced HCV-specific CTL responses induced by the genetic immunization of DNA encoding truncated HCV core proteins [68]. In another model, HBs antigen enhanced the induction of HCV-specific CTLs by DNA vaccine harboring HCV CTL epitopes [69].

Not only animal experiments, but also several human trials have been proceeding. Yutani has reported a phase I study of HCV vaccine in Japanese patients who were either nonresponders to IFN therapy ($n = 23$) or had refused treatment ($n = 3$). A peptide derived from the HCV core region amino acid residues 35–44 is capable of inducing cellular immune responses in many patients with different HLA class I-A alleles [70]. This peptide was used to develop a series of 6 vaccine injections that enhanced the peptide-specific peripheral CTL activity in 15 out of 25 patients and 12 vaccine injections that augmented peptide-specific IgG production [71]. Improvement in serum alanine aminotransferase (ALT) level (>30% decrease) was also observed in 7 out of 24 patients in the study. The results revealed that the selection of candidate peptides is crucial for developing a successful HCV vaccine.