these unsatisfactory outcomes is the high frequency of intrahepatic recurrence after curative treatment [1,2]. Intrahepatic recurrence is the result of two mechanisms: intrahepatic metastasis (IM) originating from the primary cancer, and a second primary cancer arising from multicentric carcinogenesis (MC). IM may correlate with early recurrence and poor prognosis, whereas MC is associated with relatively good prognosis [3–5].

To develop novel antitumor strategies for HCC, we have investigated the effectiveness of immune gene therapy using suicide genes and chemokine molecules, including chemokine ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1) [6–8]. CCL2/MCP-1 is a chemokine that regulates the recruitment of monocytes/macrophages to inflammatory sites and tumor tissues, as well as their activation, including lysosomal enzyme release and tumoricidal activity, and is functional in both mice and humans [9]. Transfectant-derived CCL2/MCP-1 has been found to successfully recruit monocytes into tumor tissue [10,11]. We recently described a combination strategy for the treatment of HCC, consisting of the herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) system and CCL2/MCP-1 gene delivery. We found that adenovirally delivered CCL2/MCP-1 enhanced the antitumor effects of the HSV-tk/GCV system by activating innate immune responses involving monocytes/macrophages, as well as demonstrating prolonged efficacy mediated by natural killer cells [6–8]. These experiments were performed in athymic nude mice, deficient in acquired immune responses, subcutaneously transplanted with HCC.

In the present study, we have used a liver metastasis model, in which tumor cells were infused through the portal vein (PV), to investigate whether CCL2/MCP-1 gene delivery could potentiate the antitumor effects of the suicide gene system. The results obtained indicate that the antitumor effects of the suicide gene are enhanced by codelivery of an adequate amount of CCL2/MCP-1. These antitumor effects were associated with the recruitment of monocytes/macrophages and T cells, T helper 1 (Th1) cytokine gene expression and the induction of splenocyte cytolytic activity. These findings indicate that CCL2/MCP-1 has an immunomodulatory effect on suicide gene therapy for HCC by orchestrating the innate and acquired immune responses.

Materials and methods

Animals

Male BALB/cA Jcl mice, 6–8 weeks of age, were obtained from CLEA Japan Inc. (Tokyo, Japan), maintained at constant room temperature (25 °C) and provided with free access to standard diet and tap water throughout, in accordance with institutional guidelines.

Cell lines and cell culture

The mouse HCC cell line BNL 1ME A.7R.1 (BNL) and the mouse colon cancer cell line colon 26 clone 20 (CT 26), derived from BALB/c mice (H-2d), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (30 min at 56 °C) fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, HEPES buffer, 2 mM glutamate, 1 mM penicillin/streptomycin and 0.2 mM gentamicin (Gibco, Long Island, NY, USA) at 37 °C in 5% CO_2.

Recombinant adenovirus vectors

The following replication-defective adenovirus vectors, driven by the CAG promoter [12], were prepared by recombinant DNA technology: Ad-tk, which expresses the HSV-tk gene; Ad-MCP1, which expresses the human CCL2/MCP-1 gene; and Ad-LacZ, which expresses the LacZ gene [13] (Figure 1A). Each recombinant adenovirus vector was purified and titered according to protocols supplied by the manufacturer (Takara, Ootsu, Japan). Briefly, each gene fragment (i.e. HSV-tk, CCL2/MCP-1 and LacZ) was excised from its respective insert-containing pBluescript vector and inserted into the cosmid pAxCA-wt (Takara, Ootsu, Japan), which contains essentially the full-length adenovirus type 5 genome apart from the E1 and E3 regions, thus generating the pAxCA gene (Figure 1A). The recombinant adenovirus vectors (rAds) were generated by transfecting 293 cells with pAxCA-gene and adenovirus 5-dIX DNA-terminal protein complex. These rAds were propagated in 293 cells [14], and viral stocks were prepared by standard protocols [15]. The titers of rAds were determined by the 50% tissue culture infectious dose ($TCID_{50}$) method [16].

Enzyme-linked immunosorbent assay (ELISA) for CCL2/MCP-1

Aliquots of 2.5×10^4 BNL cells were seeded in 3.0 ml of culture media in six-well tissue culture plate. After 24 h, the cells were infected with Ad-MCP1 and Ad-MCP1, together with Ad-tk, at various multiplicities of infections (MOIs). After 24 h, the media were collected from the wells, and the concentrations of CCL2/MCP-1 were determined by ELISA. Briefly, each well of a 96-well microtiter plate (Nalgene, Rochester, NY, USA) was coated with 0.05 M carbonate buffer (pH 9.6) containing monoclonal anti-human CCL2/MCP-1 antibody (ME 6.1; 1 μg/ml) overnight at 4 °C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the plates were blocked with PBS containing 1% bovine serum albumin for 1 h at 37 °C. Diluted culture medium or various concentrations of recombinant CCL2/MCP-1 were added to duplicate wells and incubated for 2 h at 37 °C. The plates were washed, incubated with rabbit anti-CCL2/MCP-1 antibodies (1 μg/ml) for 2 h at 37 °C,

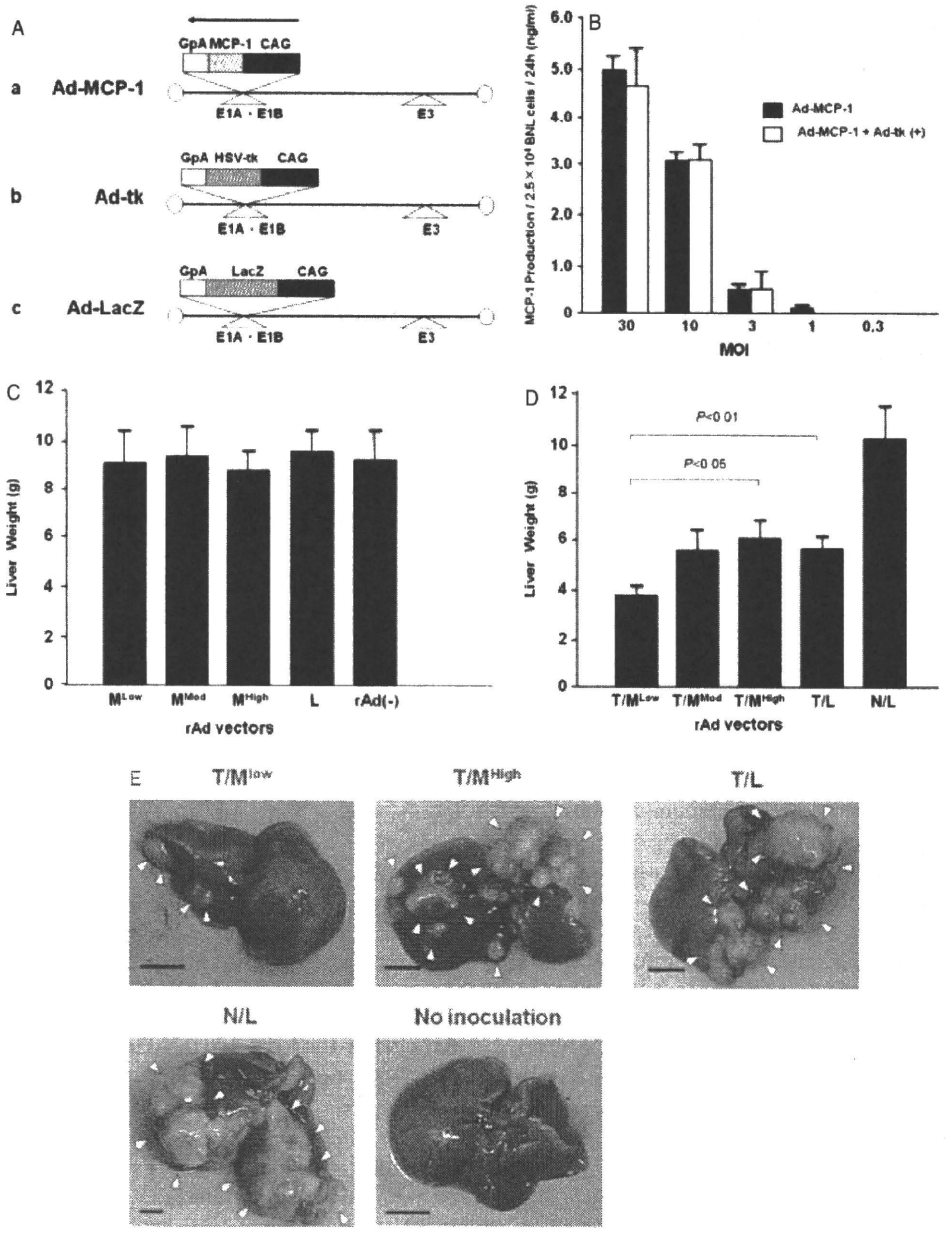

Figure 1. CCL2/MCP-1 production and antitumor effects of rAds. (A) Schematic representation of rAds expressing each gene under the control of a CAG promoter. (a) Ad-MCP1 expressing CCL2/MCP-1. (b) Ad-tk expressing HSV-tk. (c) Ad-LacZ expressing β-galactosidase gene (LacZ). Solid lines indicate the rAd genome, and the open triangle below each rAd genome represents the deletion of adenovirus early regions. The arrow shows the orientation of transcription. GpA, rabbit β-globin (A) site; CAG, CAG promoter. (B) Production of CCL2/MCP-1 by BNL cells infected with rAds at various MOIs. The CCL2/MCP-1 concentrations in culture supernatants were determined by ELISA. Data shown are the mean $\pm$ SE of three independent results. (C) Liver weight following transfer of BNL cells infected with Ad-MCP1, Ad-LacZ or PBS (−). Each mouse was injected via the PV with 1×10^6 BNL cells infected with Ad-MCP1 at various MOIs: 0.03 (M^{Low}), 0.3 (M^{Mod}) and 3 (M^{High}), and Ad-LacZ at the MOI of 3 (L), and the whole livers were weighed on day 21. (D) CCL2/MCP-1 enhancement of the antitumor effects of the HSV-tk/GCV system against intrahepatic cancer cells. Each mouse was injected via the PV with BNL cells (1×10^6) infected with Ad-tk, Ad-MCP1 and Ad-LacZ at various MOIs: Ad-tk/Ad-MCP1 = 3/0.03 (T/M^{Low}), 3/0.3 (T/M^{Mod}) and 3/3 (T/M^{High}); Ad-tk/Ad-LacZ = 3/3 (T/L); and Ad-LacZ = 6 (N/L), and the whole livers were weighed on day 21. (E) The macroscopic views of hepatic tumors (open arrowheads) in mice. Tumor growth was markedly suppressed in T/M^{Low} mice. Scale bars = 10 mm

washed again and incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:12 000; Tago, Burlingame, CA, USA) for 2 h at 37 °C. The plates were washed, aliquots of 1 mg/ml *p*-nitrophenylphosphate (Sigma, St Louis, MO, USA) in 1 M diethanolamine (pH 9.8) supplemented with 0.5 mM $MgCl_2$ were added to the wells, and the plates were incubated for 40 min at room temperature. After addition of 1 M NaCl, the optical density (at 405 nm) was assessed using an ELISA plate reader (MTP-120; Corona Electric, Ibaragi, Japan). All experiments were repeated in triplicate.

Disease model

To evaluate the direct antitumor effect of CCL2/MCP-1, 1×10^7 BNL cells suspended in 0.5 ml of culture medium were infected *in vitro* with CCL2/MCP-1 at various MOIs: 0.03 (M^{Low}), 0.3 (M^{Mod}) and 3 (M^{High}); Ad-LacZ at an MOI of 3 (L); or PBS (−). The cells were harvested after 30 min of incubation at 37 °C. BALB/c mice were anesthetized by intraperitoneal injection with sodium pentobarbital (Somnopentyl, Schering-Plough Animal Health Corporation, Kenilworth, NJ, USA), and laparotomy was performed. Each mouse was injected with 1×10^6 adenovirus-infected BNL cells in a volume of 0.2 ml PBS containing 2% FBS or 0.2 ml PBS (−) via PV on day 0. The mice were sacrificed on day 21, and their liver tissues were weighed.

To determine whether CCL2/MCP-1 can enhance the antitumor effects of the HSV-tk/GCV system, BNL cells were infected with Ad-tk, Ad-MCP1 and Ad-LacZ at various MOIs: Ad-tk/Ad-MCP1 = 3/0.03 (T/M^{Low}), 3/0.3 (T/M^{Mod}) and 3/3 (T/M^{High}); Ad-tk/Ad-LacZ = 3/3 (T/L); and Ad-LacZ = 6 (N/L). BALB/c mice were anesthetized and each was injected via portal vein with 1×10^6 adenovirus-infected BNL cells on day 0, followed by intraperitoneal injection of 75 mg/kg/day ganciclovir on days 2–6. The mice were sacrificed on day 21, and the livers were removed and weighed. Additionally, in another series of experiments, the livers removed from the mice on days 1, 3, 7 and 14 were processed for immunohistochemistry and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Simultaneously, their splenocytes were tested for cytolytic activity against ^{51}Cr-labeled BNL cells.

Histopathological and immunohistochemical analysis

Liver sections were fixed in 10% zinc-buffered formalin and stained with hematoxylin and eosin (H&E). For histological evaluation, mouse livers were harvested, embedded in tissue-Tek® OCT embedding medium (Sakura Finetek, Torrance, CA, USA) and stored at −80 °C until use, except those stained for CD31 (Abcam, Cambridge, MA, USA), arginase I (Arg-I; BD Biosciences, Franklin Lakes, NJ, USA) and inducible nitric oxide synthase (iNOS; Thermo Fisher Scientific, Fremont, CA, USA). Cryostat sections of frozen tissues were fixed in cold acetone for 10 min and rinsed three times with PBS. The tissue samples used for CD31, iNOS and Arg-I staining were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Following blocking of nonspecific tissue avidin and biotin with a blocking kit (Vector Laboratories, Burlingame, CA, USA), the slides were incubated with biotin-conjugated monoclonal antibody against Mac-1 (CD11b), CD4, CD8 (PharMingen, San Jose, CA, USA) or Arg-I or polyclonal antiserum against CD31 or iNOS for 30 min at room temperature. Biotin-conjugated rat IgG2b, kappa was used as the negative control. The reaction was visualized using a Vectastain® ABC Standard Kit (Vector Laboratories), followed by counterstaining with hematoxylin.

Real-time quantitative RT-PCR

The Frozen liver specimens containing necrotic liver tissues or tumor tissues were broken into fine pieces and total RNA was extracted from liver tissues using a ToTALLY RNA® kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using a SuperScript® first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was used for real-time quantitative PCR using the ABI PRISM 7900 (Applied Biosystems, Foster City, CA, USA) with TaqMan® Master Mix (Applied Biosystems), and primers and probes for interleukin (IL)-4, IL-10, IL-12, IL-18, interferon (IFN)γ, vascular endolthelial growth factor (VEGF)-A and 18S ribosome (sequences available on request from the authors) (Applied Biosystems). The expression of cytokine mRNA in each sample was normalized relative to that of 18S ribosome mRNA.

Cytotoxic T lymphocyte assay (^{51}Cr release assay)

The cytolytic activity of mouse spleen cells was assessed by a ^{51}Cr release assay, as described previously [17]. Briefly, each treated mouse was sacrificed on day 14, splenocytes were harvested aseptically and mashed in alpha-minimal essential medium (MEM) medium (Gibco) with 10% FBS, and suspensions of single spleen cells were prepared. Spleen cells were cultured with mitomycin C (MMC; Sigma) (400 μg/4 ml, 1 mg/ml in HBSS) treated BNL or CT26 cells in complete alpha-MEM medium containing 10% FBS and 2.5% EL-4 culture supernatant (a source of T cell growth factor) for 5 days. Target cells consisted of 3×10^5 BNL cells labeled with 0.3 mCi $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Boston, MA, USA) at 37 °C for 1 h. Effector spleen cells were incubated with 5×10^3 target cells in 96-well plates at various effector/target ratios for 4 h at 37 °C, and the ^{51}Cr released into the culture supernatants was quantified by scintillation

counting. Percent specific cytotoxicity was calculated according to the equation: [100 × (experimental release – spontaneous release)/(maximum release – spontaneous release)]. Spontaneous release was defined as the ^{51}Cr in the supernatant of target cells incubated for 4 h, and maximum release was defined as ^{51}Cr in the supernatant of target cells treated with 2% Triton-X. Experiments were performed three times and the results expressed as the mean ± SE. Tumor specificity was determined based on differences between BNL and CT 26 cells. All results presented are the means of triplicate assays.

Results

CCL2/MCP-1 production of recombinant adenoviruses *in vitro*

The production of CCL2/MCP-1 was evaluated by measuring the concentrations in culture media of BNL cells infected with varying MOIs of Ad-MCP1 and Ad-MCP1 plus Ad-tk by ELISA (Figure 1B). The production of CCL2/MCP-1 by cells infected with Ad-MCP1 increased in proportion to the MOI. Importantly, its production by Ad-MCP1 infected cells was not changed when these cells were further infected with Ad-tk (Ad-MCP1 plus Ad-tk), indicating that CCL2/MCP-1 production by Ad-MCP1 was not influenced by coinfection with Ad-tk in BNL cells. In addition, the functional properties of CCL2/MCP-1 produced by this rAd were defined previously [6–8].

Intrahepatic tumor development following transfer of HCC cells infected with recombinant adenoviruses

To evaluate the direct antitumor effect of CCL2/MCP-1 in an immunocompetent mouse model of IM, mice were injected via the PV with BNL cells infected with Ad-MCP1 or Ad-LacZ at various MOIs (Figure 1C). When whole livers were weighed on day 21, the weights of M^{Low} ($n = 4$), M^{Mod} ($n = 7$) and M^{High} ($n = 6$) were comparable to those of L ($n = 4$) mice, indicating that delivery of CCL2/MCP-1 gene did not promote or suppress the growth of tumor cells in this model.

To determine whether CCL2/MCP-1 gene delivery can potentiate the antitumor effects of the HSV-tk/GCV system, mice were injected with BNL cells infected with rAds (Ad-tk, Ad-MCP1 and Ad-LacZ) at various MOIs as described in the Materials and methods. Whole livers were weighed on day 21, and the weights of T/M^{Low} ($n = 14$), T/M^{Mod} ($n = 12$), T/M^{High} ($n = 11$) and T/L ($n = 10$) mice were compared with those of N/L ($n = 10$) mice. The reduction in liver weight for the T/L mice was a result of the HSV-tk/GCV system alone, and those for T/M^{Low}, T/M^{Mod} and T/M^{High} were a result of treatment in combination with CCL2/MCP-1. Mean ± SEM liver weight was significantly lower in T/M^{Low} mice than in T/L mice (3.91 ± 0.36 g versus 5.80 ± 0.58 g; $p < 0.01$) (Figures 1D and 1E) as a result of the reduced growth of implanted tumor cells in the former. By contrast, the increase in liver weight was not suppressed in T/M^{Mod} and T/M^{High} mice whose tumor cells had been treated with higher titers of Ad-MCP1. Thus, only low level CCL2/MCP-1 provided additional antitumor effects and these results indicate that delivery of adequate amounts of Ad-MCP1 enhanced the antitumor effects of the HSV-tk/GCV system against intrahepatic tumor cells.

Serial analysis of liver histology following tumor cell transfer

To monitor the course of tumor development following HCC cell transfer, mouse livers were harvested on days 1, 3, 7 and 14. Livers harvested on day 1 from all groups of mice injected with BNL cells showed multiple white patches on their surfaces. Histologically, hepatocyte degeneration and necrosis were observed in these lesions, suggesting that the reduction of PV flow by transferred tumor cells induced focal ischemic necrosis in the livers (Figure 2, closed arrowheads). Although the extent of necrosis was similar among all groups, inflammatory cell infiltration in the area of necrosis was greater in T/M^{Low}, T/M^{High} and T/L than in N/L mice. On day 3, cellular infiltration disappeared, and tumor cell growth was observed in areas surrounding the necrotic regions. On day 7, proliferation of viable tumor cells surrounding the necrosis was seen in the livers of N/L mice, with the necrotic tissues completely replaced by tumor cells. Tumor growth was moderately inhibited in T/L mice and greatly inhibited in T/M^{Low} mice. There was no difference between T/L and T/M^{High} mice (not shown). On day 14, the necrotic areas were almost absorbed in all mice. In N/L mice, the tumor cells grew progressively and the tumor masses became larger. Tumor volume was relatively lower in T/L than in N/L mice, although there was no significant difference between T/L and T/M^{High} mice. The greatest degree of tumor growth inhibition was observed in T/M^{Low} mice (Figure 2).

Recruitment of immune cells in liver

To evaluate the involvement of immune responses in the CCL2/MCP-1 associated enhancement of the antitumor effects of rAd expressing HSV-tk, we assessed the recruitment of Mac-1^+ monocytes/macrophages and $CD4^+$ and $CD8^+$ T lymphocytes immunohistochemically (Figure 3).

T/M^{Low} and T/M^{High} mouse liver tissues harvested on day 1 showed marked infiltration of Mac-1^+ cells in the necrotic areas induced by tumor cell injection (Figure 3A). Quantitative morphometric analysis showed that the numbers of Mac-1^+ cells were significantly higher in liver tissues of T/M^{Low} and T/M^{High} mice [mean ± SEM of 40 high power (×400) fields of necrotic liver

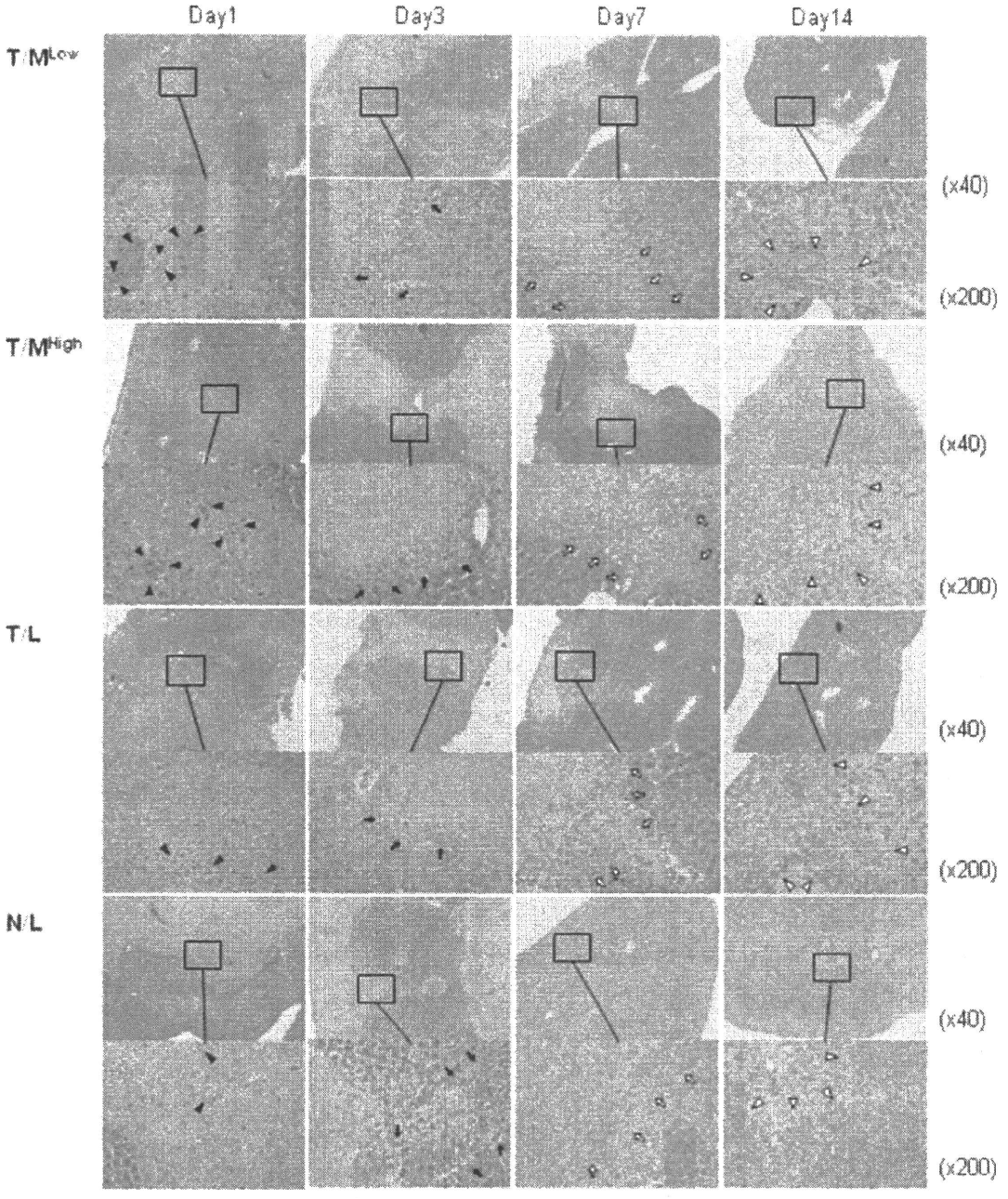

Figure 2. Serial analysis of liver histology following tumor cell transfer. Mouse liver tissues were harvested on days 1, 3, 7 and 14, and stained with H&E. On day 1, all mice injected with BNL cells showed multiple white patches on the liver surface (not shown). Histologically, hepatocyte degeneration and necrosis were observed in these lesions, suggesting that the reduction of PV flow by transferred tumor cells induced focal ischemic necrosis in the livers. The area of necrosis significantly infiltrated with inflammatory cells (closed arrowheads) was higher in T/M^{Low}, T/M^{High} and T/L mice than in N/L mice. On day 3, cellular infiltration disappeared and tumor cell growth (closed arrows) was detected in areas surrounding the necrotic regions. On day 7, tumor tissue enlarged and replaced the necrotic areas (open arrows). On day 14, the necrotic areas disappeared and tumor nodules eventually formed (open arrowheads). Original magnifications $\times 40$ and $\times 200$

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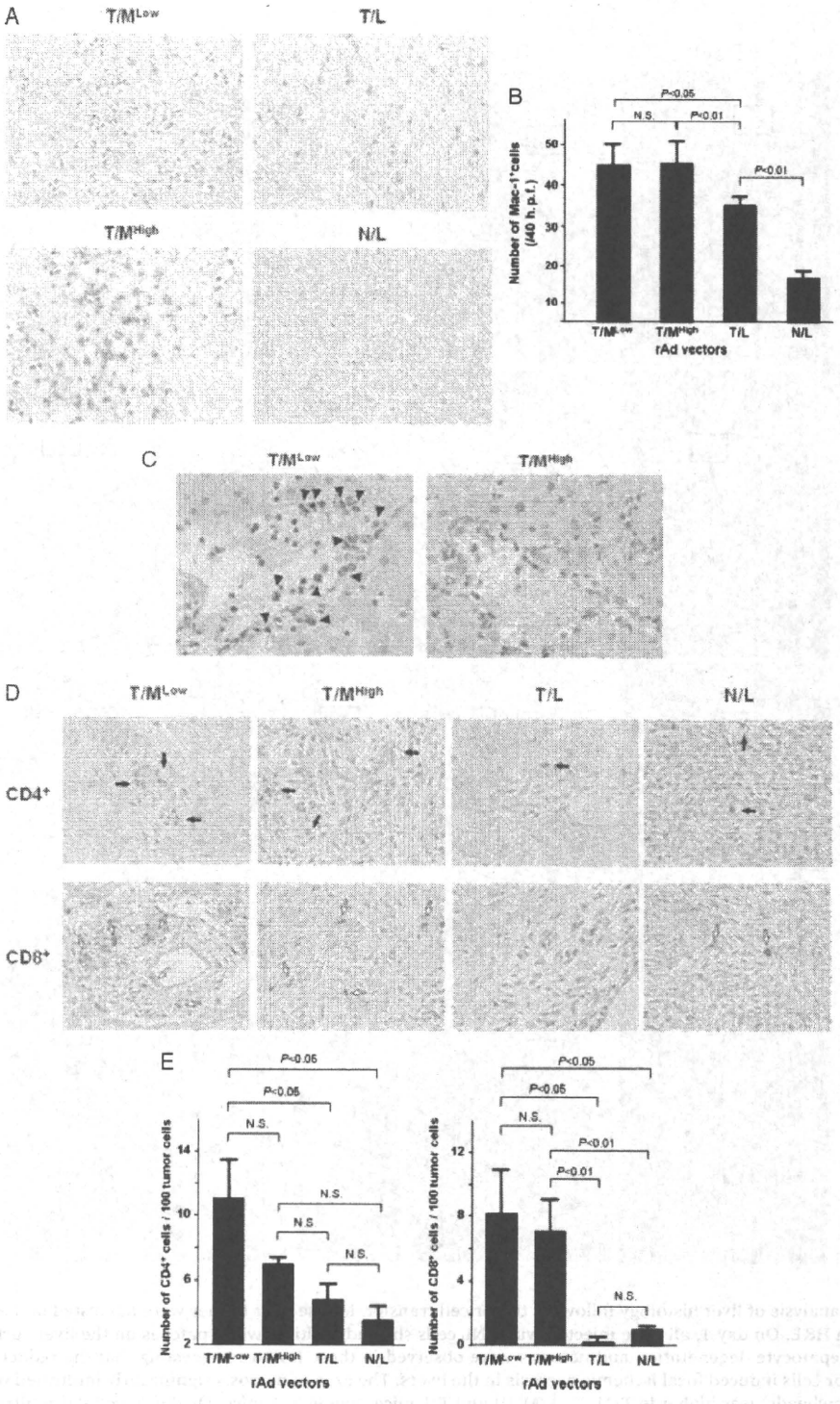

Figure 3. Immunohistochemical evaluation of monocyte/macrophage (A–C) and T cell (D, E) recruitment into liver tissues. (A) Monocyte/macrophage detection using anti-Mac-1 monoclonal antibody. Original magnification ×400. (B) Quantitative morphometric analysis of Mac-1$^+$ cells. (C) Immunohistochemical evaluation of the polarization towards M1 phenotype of recruited monocytes/macrophages using antibody against iNOS (closed arrowheads). (D) CD4$^+$ (closed arrows) and CD8$^+$ (open arrows) T cell detection. Original magnification ×400. (E) Quantitative morphometric analysis of CD4$^+$ and CD8$^+$ T cells

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tissues: 46.5 ± 3.7 and 46.9 ± 3.7; $p < 0.05$ and $p < 0.01$, respectively] compared to T/L mice (35.2 ± 2.4). Macrophages can be activated not only by CCL2/MCP-1, but also by tumor cells treated with Ad-tk [6]. In T/L mice, these cells may induce moderate infiltration of Mac-1^+ cells (Figure 3B). These findings indicate that the codelivery of the HSV-tk and CCL2/MCP-1 genes was associated with a higher degree of infiltration of Mac-1^+ monocytes/macrophages during the initial period of tumor development. Additionally, to investigate whether the recruited monocytes/macrophages were polarized towards the M1 or M2 phenotype, we performed immunohistochemical analysis using antibodies against iNOS (M1) and Arg-I (M2) [17,18] (Figure 3C). The proportion of iNOS$^+$ (M1 subset) cells among the inflammatory cells was significantly higher in T/M^{Low} than in T/M^{High} mice [mean ± SEM number (per 100 inflammatory cells) of eight high power (×400) fields of necrotic liver tissues: 23.0 ± 2.7 and 10.8 ± 2.5; $p < 0.01$, respectively]. Arg-I$^+$ (M2 subset) cells were not specifically detected, most likely as a result of the large amounts of the enzyme present in liver tissues.

Similarly, liver tissues obtained on day 14 after HCC cell transfer were immunohistochemically analyzed for immune cell infiltration. In both T/M^{Low} and T/M^{High} mice, the tumor foci were heavily infiltrated by CD4$^+$ and CD8$^+$ T cells (Figure 3D). Quantitative morphometric analysis showed that the numbers of CD4$^+$ and CD8$^+$ T cells were higher in T/M^{Low} [mean ± SEM number (per 100 tumor cells) of eight high power (×400) fields of liver tissues: 11.1 ± 2.5 and 8.1 ± 2.7; $p < 0.05$ and $p < 0.05$, respectively] and T/M^{High} (7.1 ± 1.9 and 7.1 ± 0.7; not significant and $p < 0.01$, respectively) mice than in T/L mice (4.8 ± 1.1 and 0.3 ± 0.3, respectively) (Figure 3E). These results suggest that the antitumor activities in T/M^{Low} mice may be mediated not only by the activation of macrophages during the initial period of tumor development, but also by the induction of T cell-mediated immune responses during later periods.

Cytokine gene expression in liver

Mice injected with adenovirus-infected HCC cells were sacrificed on day 1 and their liver tissues were analyzed by quantitative real-time RT-PCR for the expression of mRNA encoding the cytokines IL-4, IL-10, IL-12, IL-18 and IFN-γ. IL-12 mRNA expression was induced to a greater extent in T/M^{Low} mice than in the other groups ($p < 0.05$). IL-18 mRNA expression tended to be high in the mice treated with CCL2/MCP-1, although these differences were not statistically significant (Figure 4). By contrast, IL-4, IL-10 and IFN-γ mRNA was not detected in any samples. These data suggest that infiltrating monocytes/macrophages induced by CCL2/MCP-1 may be activated to enhance the Th1-polarized responses that contribute to tumor immunity.

Microvessels in HCC

CCL2/MCP-1 has been shown to be associated with angiogenesis [19,20]. To understand the basis of the different antitumor effects observed in T/M^{Low} and T/M^{High} mice, we immunohistochemically stained microvessels within HCCs for CD31. We found that CD31$^+$ microvessels were markedly increased in 7-day tumor tissues of T/M^{High} mice relative to T/M^{Low} and T/L mice (Figure 5A). These results suggest that angiogenesis induced by large amounts of CCL2/MCP-1 may contribute to tumor growth in T/M^{High} mice.

VEGF gene expression in liver

Liver samples harvested on day 3 were analyzed for the expression of VEGF-A mRNA, which encodes an angiogenic factor that may promote tumor growth. Quantitative real-time RT-PCR showed that VEGF-A gene expression was induced to a greater extent in T/M^{High} mice (Figure 5B), suggesting that the CCL2/MCP-1 enhancement of antitumor effects may be offset by VEGF-induced angiogenesis.

Cytotoxic activities of splenocytes

To assess the cytotoxic activities of immune cells derived from mice injected with adenovirus-infected tumor cells, isolated and pulsed splenocytes were incubated with ^{51}Cr-labeled BNL cells in a standard 4-h cytotoxicity assay (Figure 6). Induction of cytotoxic T lymphocytes (CTLs) specific for BNL cells was higher in T/M^{Low} and T/M^{High} mice than in T/L (not significant) and N/L ($p < 0.01$) mice, and there was no significance difference between T/M^{Low} and T/M^{High} mice. Because the immunogenicity of viral vectors or transgene products is known to induce the unfavorable host immune responses, the detection of antitumor CTL activities may be influenced by the responses against rAd vector and HSV-tk [21–23]. Especially, CTL responses against HSV-tk appear to be induced in T/L, T/M^{Low} and T/M^{High} mice. Collectively, the data suggest that cytotoxic activity of CTLs may be enhanced by the codelivery of a suicide gene and CCL2/MCP-1, consistent with their *in vivo* enhancement of antitumor effects.

Discussion

In the present study, we have shown that combination gene therapy, using the HSV-tk/GCV system and CCL2/MCP-1 gene delivery, was effective for the treatment of HCC in a mouse model of IM. Delivery of an adequate amount of CCL2/MCP-1 enhanced the antitumor effects of the HSV-tk/GCV system against intrahepatic tumor cells. Necrotic areas induced by HCC tumor cell injection showed marked infiltration of iNOS$^+$

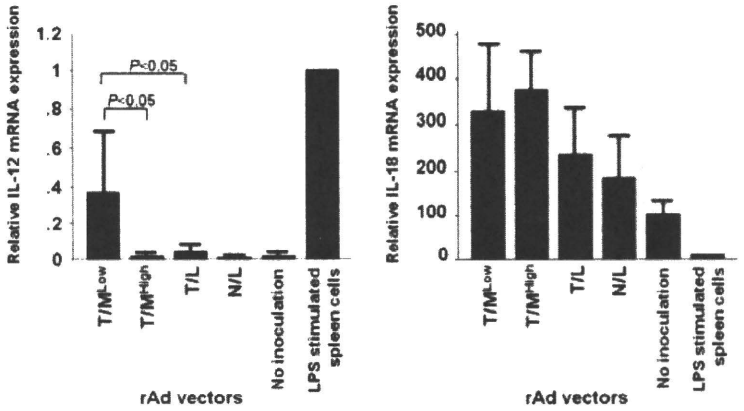

Figure 4. Real-time quantitative RT-PCR for IL-12 and IL-18 mRNA expression in the liver on day 1. IL-12 gene expression was significantly higher in T/M^{Low} mice than in the other groups ($p < 0.05$)

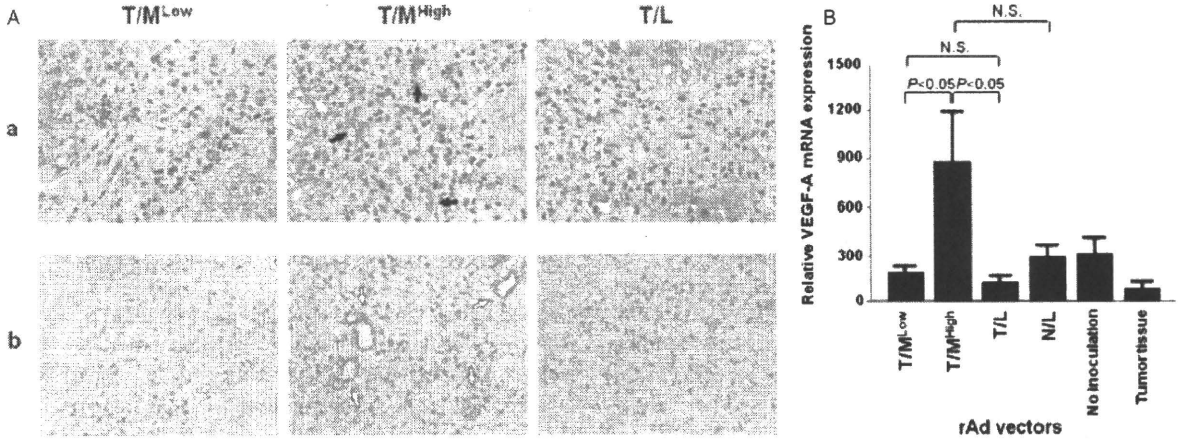

Figure 5. Evaluation of tumor angiogenesis. (A) Morphometric analysis of microvessels in tumor tissues using H&E staining and CD31 immunohistochemical analysis. (a) Representative H&E stained histological sections of day 7 tumor tissues showing intratumoral microvessels containing red blood cells (closed arrow); endothelial cells were not identified. (b) Representative CD31 immunohistochemical staining showing endothelial cell proliferation in tumor tissues (open arrow). Original magnification ×400. (B) Real-time quantitative RT-PCR for VEGF-A mRNA expression in liver on day 3

monocytes/macrophages and IL-12 production on day 1, and the tumor foci showed heavy infiltration by CD4$^+$ and CD8$^+$ T cells on day 14. CTLs specific for BNL cells were induced in mice treated with CCL2/MCP-1. By contrast, the expression of the angiogenic factor VEGF-A was significantly increased in mice treated with a large amount of CCL2/MCP-1. Collectively, these results suggest that the delivery of an adequate amount of CCL2/MCP-1, in conjunction with the HSV-tk/GCV system, may display beneficial antitumor effects, preventing the intrahepatic metastasis of HCC cells.

In the development of this model, we injected 1×10^6 tumor cells infected with recombinant adenoviruses into the portal vein because the injection of fewer cells (e.g. 10^5) resulted in greatly diminished frequencies of metastasis in the mice. The injection of large numbers of cells, however, may have caused the embolization of cell aggregates in the portal vein, which may have contributed to the induction of ischemic necrosis in the liver tissues. The resultant ischemic death of liver cells may be recognized by immune cells including macrophages, and may result in macrophage activation and the local release of cytokines and chemokines. However, when the mice were injected with control tumor cells (N/L), we observed little infiltration of immune cells, including macrophages and CD4$^+$ and CD8$^+$ T cells, and these mice developed the largest amounts of tumor tissues. These results indicate that any unfavorable effects as a result of ischemic cell death were minimal for the development of intrahepatic metastasis in this model.

This model would be more relevant if ganciclovir treatment was delayed to allow the establishment of tumors. Therefore, we performed the additional experiments with ganciclovir treatment at delayed time point, day 3. Although there was a trend for small amount of MCP-1 to enhance the antitumor effects of the HSV-tk/GCV system, as seen in the experiments on day 1, these differences did not reach statistical significance [T/M^{Low}: 7.64 ± 1.25 ($n = 10$); T/M^{Mod}: 9.24 ± 0.77 ($n = 5$); T/M^{High}: 9.65 ± 1.06 ($n = 8$); T/L: 10.51 ± 1.79

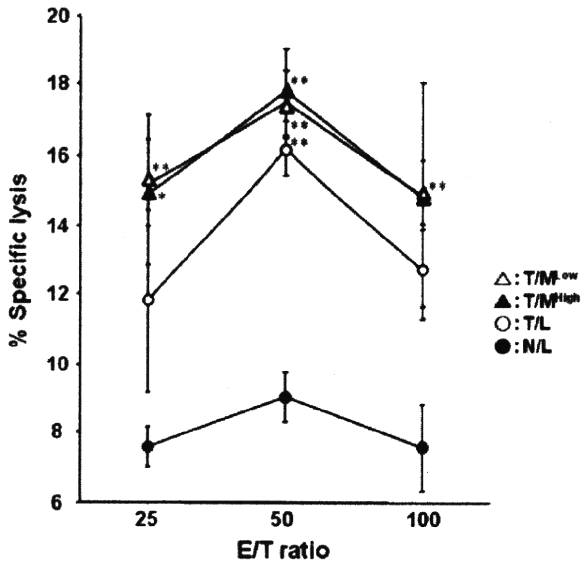

Figure 6. Cytotoxic activities of splenocytes. Splenocytes harvested on day 14 from individual mice stimulated with MMC-treated BNL cells for 7 days were tested in a standard 4-h cytotoxicity assay with ^{51}Cr-labeled target (BNL) or control (CT26) cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to N/L mice

($n = 7$); and N/L: 13.94 ± 1.16 ($n = 5$)]. Consequently, the experiment in which ganciclovir was added 3 days after tumor inoculation failed to show a significant antitumor effect. The weakness of this approach may be still the low relevance of the tumor model. The reason is that MCP-1 gene expression by rAds may not be sufficient to enhance antitumor effect at day 3 because the transgene expression gradually diminished with the tumor growth. In our previous studies, MCP-1 production reached peak level on day 2 and decreased after day 3 [6].

Mice treated with small amounts of CCL2/MCP-1 showed enhancement of antitumor effects. The amount of CCL2/MCP-1 delivered, however, was not correlated with monocyte/macrophage accumulation. Although activated monocytes/macrophages are indicative of the potential to eliminate tumor cells [24–26], infiltrating macrophages may enhance tumor growth by secreting growth and angiogenic factors, including VEGF [26–28]. Immunohistochemical analysis of CD31 revealed that microvessels in HCCs were increased in the mice treated with large amounts of CCL2/MCP-1. We also observed a close correlation between the amounts of CCL2/MCP-1 delivered and the levels of VEGF expression. These findings suggest that large amounts of CCL2/MCP-1 may recruit macrophages to induce tumor cell killing and, simultaneously, to facilitate tumor growth, probably by promoting angiogenesis, thus resulting in a reduction of antitumor effects.

CCL2/MCP-1 is a member of the CC chemokine superfamily that promotes the migration of macrophages/monocytes, T lymphocytes, natural killer cells and natural killer T cells not only to sites of inflammation, but also to tumor tissues, which may contribute to the inhibition of tumor growth [29–31]. In addition, the production of CCL2/MCP-1 by tumor tissues has been reported to be associated with favorable prognoses in human pancreatic cancer [31] and neuroblastoma [30]. By contrast, CCL2/MCP-1 may promote tumor growth by chemoattracting tumor-associated macrophages for tumor angiogenesis, or by acting on tumor cells as an autocrine growth factor [29,32,33]. Consistent with this notion, a Japanese study of 135 breast cancer patients found that the women with high levels of tumor-associated CCL2/MCP-1 showed a significantly shorter relapse-free survival [34]. Taken together, the biological and immunological effects of CCL2/MCP-1 appear to vary greatly depending on the diverse microenvironments of cancer tissues.

Two major types of activated macrophages have been described: M1 (classical) and M2 (alternative) [35–38]. M1 macrophages, which play a critical role in the development of antitumor immunity, are characterized by high IL-12 and low IL-10 production. By contrast, M2 macrophages produce reduced amounts of IL-12 but higher levels of Il–10. We found that IL-12 expression was significantly increased in mice treated with a small amount of CCL2/MCP-1 but not in mice treated with a large amount of CCL2/MCP-1, despite the marked infiltration of monocytes/macrophages in the latter. In addition, members of the MCP family have been reported to dose-dependently inhibit IL-12 production by antigen-presenting cells (APCs) [39,40]. Because of the different local concentrations of CCL/MCP-1, we hypothesized that the M1/M2 ratio of recruited monocytes/macrophages may differ in T/M^{Low} and T/M^{High} mice. Indeed, we found that the proportions of M1 cells among infiltrating cells were significantly higher in T/M^{Low} than in T/M^{High} mice. Therefore, M1 monocyte/macrophage polarization may be suppressed in mice treated with large amounts of CCL2/MCP-1, resulting in the reduction of antitumor immunity and the promotion of tumor growth.

Significant tumor infiltration of CD4$^+$ and CD8$^+$ T cells 14 days after transfer was observed in mice treated with the HSV-tk/GCV system plus CCL2/MCP-1. Local secretion of CCL2/MCP-1 by tumor cells may lead to the recruitment and activation of antigen-presenting monocytes/macrophages [41,42]. Once attracted to the tumor tissues, these APCs may ingest pathogenic antigens and transport them to local lymphoid organs, where the antigens are presented to naive T cells, thus establishing a T cell-mediated antitumor response [43]. Tumor growth may thus be impeded by tumor antigen-specific CD4$^+$ and CD8$^+$ T cells.

Although the data obtained in the present study appear to be promising, several problems remain to be solved before clinical application. Our liver metastasis model using a mouse HCC cell line may not be comparable to intrahepatic metastasis of HCC in human patients. However, HCC patients treated by nonsurgical procedures, including percutaneous radiofrequency ablation therapy and transcatheter arterial chemotherapy [44,45], could also be administered rAds to reduce the incidence of

intrahepatic recurrence and metastasis. The present study demonstrated that, in a mouse model, there is a negative impact on tumor development in the presence of a low level of CCL2/MCP-1, whereas high levels of the protein complicate the situation by having a positive impact on tumor growth (i.e. a balance is required). The therapeutic effects may vary with different tumors. The direct correlation between the overexpression of VEGF in tumor cells and tumor angiogenesis has been demonstrated previously [46], and large amount of CCL2/MCP-1 might be less effective in the treatment of hypervascular tumors such as HCC. However, other cancers resistant to anti-angiogenic drug (e.g. pancreatic cancer) [47,48], probably do not need a good blood supply for tumor growth. In the treatment of hypovascular tumors that are resistant to anti-angiogenic drug, CCL2/MCP-1 may enhance the antitumor effects via activation of M1 macrophages.

Additionally, in the present study, we did not perform *in vivo* delivery experiments of the vectors to existing tumors. There would be many complicated factors affecting the delivery of HSV-tk and CCL2/MCP-1 genes in therapeutic approaches [49,50]. Intra-arterial administration of rAds may result in the induction of immunogenicity or cytotoxicity, especially when spread via blood flow. Extremely high-dose rAds have been found to cause severe unexpected side-effects [51]. To overcome these problems, highly tumor-specific promoters may be needed. In our previous studies, human alpha-fetoprotein (AFP) promoters specific for liver cancer cells were used in an immunodeficient nude mouse models [6,52]. A reporter gene was specifically expressed in AFP producing tumors that were xenografted subcutaneously and disseminated in the liver and lung. However, HSV-tk gene expression was not enhanced sufficiently to kill established tumor cells [53] because the transcriptional activity of AFP promoter was relatively low. Furthermore, neither promoters, nor delivery systems were found to be specific for the BNL mouse tumor cell line. Although better methods of tumor-specific gene delivery and expression are needed, the use of *ex vivo* infection techniques has been found to reproduce tumor specific gene expression *in vivo*.

Conclusions

Although problems with rAds remain to be resolved before clinical application, the results obtained in the present study suggest that a new strategy, consisting of immune gene therapy accompanied by a suicide gene system, can be used to treat HCC and tumors of other lineages.

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Original Article

Efficacy and safety of double filtration plasmapheresis in combination with interferon therapy for chronic hepatitis C

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Aim: Efficacy and safety of double filtration plasmapheresis (DFPP) for chronic hepatitis C were prospectively analyzed in Japanese clinical settings.

Methods: All patients who received DFPP in combination with interferon (IFN) therapy for chronic hepatitis C were serially recruited at 36 institutions between April 2008 and July 2009 in Japan.

Results: A total of 239 patients were analyzed for the safety of DFPP and 206 patients for the efficacy. Of the 206 patients, 181 patients were treated with DFPP in combination with pegylated interferon plus ribavirin (PEG-IFN/RBV + DFPP). Among the 181 patients, 60 patients (33.1%) were treatment-naïves, 35 (19.3%) relapsers and 62 (34.3%) non-responders. Complete early virological response (cEVR) in patients treated with PEG-IFN/RBV + DFPP was achieved in 57.5% overall, 70.0% in treatment-naïves, 57.1% in relapsers and 41.9% in non-responders. In patients with previous PEG-IFN/RBV therapy, cEVR were found in 63.0% of relapsers and 18.9 % of non-responders, and cEVR in patients with other than PEG-IFN/RBV therapy as previous IFN therapy, relapsers and non-responders was 37.5% and 76.0%, respectively. Adverse events were found in 55 patients (23.0%). Serious adverse events were found in four patients (1.7%) who showed puncture-site injury. Adverse events were related to female sex, but not related to age, and DFPP could be performed safely.

Conclusion: The cEVR results in this study suggest that high rates of sustained virological response can be achieved in retreated and treatment-naïve patients using DFPP in combination with PEG-IFN/RBV therapy. Results indicate that this therapy could be safely conducted, even in elderly patients.

Key words: complete early virological response, double filtration plasmapheresis, elderly, interferon therapy, retreated patients, treatment-naïve patients

INTRODUCTION

CHRONIC HEPATITIS C patients with genotype 1b and a high viral load are known to be the most refractory to interferon (IFN) therapy. In Japan, this type of patient accounts for approximately 70% of chronic hepatitis C cases, and various treatment methods have been investigated to improve the therapeutic effects. Currently, the first choice therapy for the refractory patients is 48-week administration of pegylated IFN (PEG-IFN) plus ribavirin (RBV). However, even with this therapy, sustained virological response (SVR) is achieved in at most 50% of the patients.[1,2] Thus, while advances are being made in the development of drugs to replace IFN or for use in combination with IFN,[1] other attempts are being made to physically remove hepatitis C virus (HCV) from the blood of chronic hepatitis C patients.

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Sakai *et al.* investigated the possibility of enhancing the therapeutic effects of IFN by removing HCV from the blood using apheresis.[4,5] Yamashita *et al.* later reported that use of double filtration plasmapheresis (DFPP) in combination with IFN/RBV achieved earlier virological response compared to conventional IFN/RBV therapy.[6] In addition, the Virus Reduction Therapy Study Group reported in 2007, that higher SVR was obtained with DFPP in combination with PEG IFN/RBV than with conventional PEG IFN/RBV therapy.[7]

Double filtration plasmapheresis was approved for use in chronic hepatitis C patients in April 2008 by the Japanese Ministry of Welfare. In order to investigate the efficacy and safety of this therapy in a larger number of patients, we report here the prospective analyses for DFPP in combination with PEG IFN/RBV through 12 weeks from the start of therapy after approval of the therapy.

METHODS

DFPP in combination with IFN therapy

PATIENTS WERE SERIALLY recruited at 36 institutions in Japan between April 2008 and July 2009. All patients were treated with DFPP in combination with IFN therapy. According to the policy of the Declaration of Helsinki, the detailed explanation of the treatment for this study had been done for each patient and had been fully understood before treatment. DFPP was performed using Plasmaflo OP (Asahi Kasei Kuraray Medical, Tokyo, Japan) as the first filter, and Cascadeflo EC-50W (Asahi Kasei Kuraray Medical) as the second filter. For each session, the final volume of treated plasma was 50 mL/kg. No restrictions were placed on the type of IFN.

The following were analyzed: sex, age, weight, histology of liver biopsy and history of IFN therapy. Laboratory test values investigated were alanine aminotransferase (ALT), aspartate aminotransferase (AST), platelet count and fibrinogen. With respect to the DFPP procedure, the number of sessions of DFPP, the treated plasma volume per session and the type of anticoagulant used were recorded. With regard to IFN therapy, the type of IFN and RBV used and the status of their usage were also investigated.

Measurement of HCV RNA

The quantity of HCV RNA was measured using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan; detection limit: 1.2 log IU/mL).

Viral load reduction and virological response

Hepatitis C virus RNA levels were investigated before the start of treatment, and at 4 and 12 weeks after the start of treatment. The reduction in viral load was calculated as the difference between viral load before treatment and viral load after 4 weeks of treatment. To compare virological response to values measured in the earlier study[7] in which measurements were performed using the Amplicore HCV Monitor method (Roche Diagnostics; detection limit: 0.05 KIU/mL = 1.7 log IU/mL), HCV RNA levels of less than 1.2 log IU/mL were regarded as negative in this study. A HCV RNA level of less than 1.2 log IU/mL after 4 weeks of treatment was regarded as a rapid virological response (RVR), and a HCV RNA level of less than 1.2 log IU/mL after 12 weeks of treatment was regarded as a complete early virological response (cEVR).

Efficacy of treatment

Efficacy was assessed by evaluating the reduction in viral load after 4 weeks of treatment, RVR, and cEVR in patients with serogroup 1 (genotype 1b) and high viral load (HCV RNA ≥5.0 log IU/mL). Intention to treat (ITT) and per protocol set (PPS) analyses were performed. ITT was performed in all subjects evaluated for efficacy. PPS was performed in patients treated with DFPP in combination with PEG IFN/RBV, who showed an adherence to PEG IFN of 80% or more and RBV of 60% or more, and who underwent three or more sessions of DFPP. Factorial analysis of cEVR was performed using patient background characteristics.

Adverse events and safety of treatment

Symptoms, diseases, abnormal laboratory values occurring between initial DFPP treatment and the day after final DFPP and for which a causal relationship with DFPP could not be ruled out were regarded as adverse events. For laboratory values, platelet counts and fibrinogen levels were assessed before the start of treatment and pre-treatment of each DFPP.

Safety was assessed in all recruited subjects based on adverse events and changes in platelet counts and fibrinogen levels during DFPP treatment. Adverse events were subjected to factorial analysis by patient background characteristics.

Statistical analyses

Fibrinogen levels and platelet counts were analyzed using repeated-measures ANOVA and the Bonferroni cor-

rection. Factorial analyses of cEVR rates and the adverse events by patient background characteristics were performed using Fisher's exact test.

RESULTS

Outline of patient population and background characteristics

A TOTAL OF 239 patients were recruited and treated with DFPP in combination with IFN therapy. An outline of the patient population is shown in Figure 1. Safety was analyzed in all 239 patients and efficacy was analyzed in 206 patients. Of the 206 patients, 181 were treated with DFPP in combination with regular PEG IFN/RBV therapy (PEG IFN/RBV + DFPP), and the remaining 25 patients were treated with IFN-β and DFPP therapy initially and then with PEG IFN/RBV therapy (IFN-β + DFPP then PEG IFN/RBV). Because the dose, period and frequency of IFN-β administration in IFN-β + DFPP then PEG IFN/RBV were quite different in each patient, we did not evaluate the efficacy in this group. Among 181 patients treated with PEG IFN/RBV + DFPP, 60 patients (33.1%) were treatment-naïve, 35 (19.3%) relapsers and 62 (34.3%) non-responders (Table 1). With regard to previous IFN therapy, 73 patients had previously been treated with PEG IFN/RBV therapy and 42 patients with other than PEG IFN/RBV therapy (Fig. 1).

Efficacy of treatment

For 181 patients treated with PEG IFN/RBV + DFPP, the reduction in viral load after 4 weeks of treatment in all

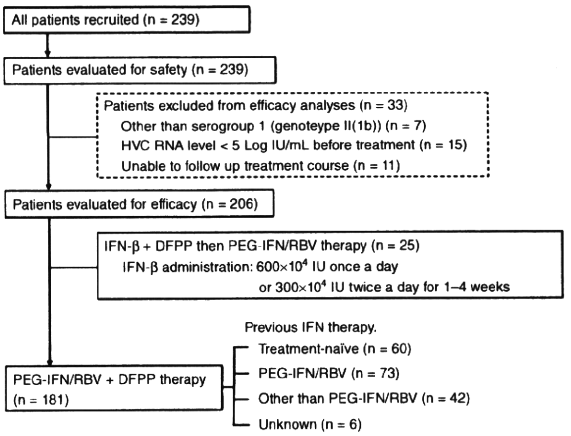

Figure 1 Outline of patient population. DFPP, double filtration plasmapheresis; HCV, hepatitis C virus; IFN, interferon; PEG, pegylated; RBV, ribavirin.

Table 1 Background characteristics of patients treated with PEG IFN/RBV + DFPP ($n = 181$)

Sex (M/F)	90/91
Age (years)	59 (24–75)†
Weight (kg)	59.0 (38.0–108.0)†
Liver biopsy	
Grading (0/1/2/3)	2/30/36/7
Staging (0/1/2/3/4)	3/20/35/13/4
Hepatitis C virus RNA level (log IU/mL)	6.4 (5.0–7.7)†
ALT (IU/L)	47 (10–294)†
AST (IU/L)	47 (12–225)†
PLT ($\times 10^4/\mu L$)	14.1 (4.4–36.8)†
Fibrinogen (mg/dL)	234 (121–430)†
Outcome of previous IFN therapy, *n* (%)	
Treatment-naïve	60 (33.1%)
Relapser	35 (19.3%)
Non-responder	62 (34.3%)
Outcome of previous IFN therapy: unknown	18 (9.9%)
Previous IFN therapy and its outcome: unknown	6 (3.3%)

†Data are presented as median (minimum–maximum).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLT, platelet count; DFPP, double filtration plasmapheresis; IFN, interferon; PEG, pegylated; RBV, ribavirin.

patients, treatment-naïve patients, relapsers and non-responders was 2.79 ± 1.85, 3.21 ± 1.81, 3.02 ± 1.40 and 2.19 ± 1.91 log IU/mL, respectively (Fig. 2a). RVR in all patients, treatment-naïves, relapsers and non-responders was 14.9% (27/181), 20.0% (12/60), 8.6% (3/35) and 12.9% (8/62), respectively, and cEVR was 57.5% (104/181), 70.0% (42/60), 57.1% (20/35) and 41.9% (26/62), respectively (Fig. 3a). In PPS analysis, cEVR was 63.7% (93/146), 78.7% (37/47), 64.5% (20/31) and 46.0% (23/50), respectively. Factorial analyses by patient background characteristics showed significant decrease in cEVR rate in patients with platelet counts of less than $10 \times 10^4/\mu L$ ($P < 0.001$), however, no difference was shown whether patients were older than 60 years or not ($P = 0.541$) (Table 2).

Among 73 patients with previous PEG IFN/RBV therapy, the reduction in viral load after 4 weeks for all patients, relapsers and non-responders was 2.24 ± 1.69, 3.05 ± 1.36 and 1.61 ± 1.63 log IU/mL, respectively (Fig. 2b). The rate of cEVR in all patients, relapsers and non-responders was 41.1% (30/73), 63.0% (17/27) and 18.9% (7/37), respectively (Fig. 3b). In PPS analysis, cEVR was 45.8% (27/59), 65.4% (17/26) and 25.0% (7/28), respectively.

Among 42 patients with other than PEG IFN/RBV therapy (e.g. IFN monotherapy) as previous IFN

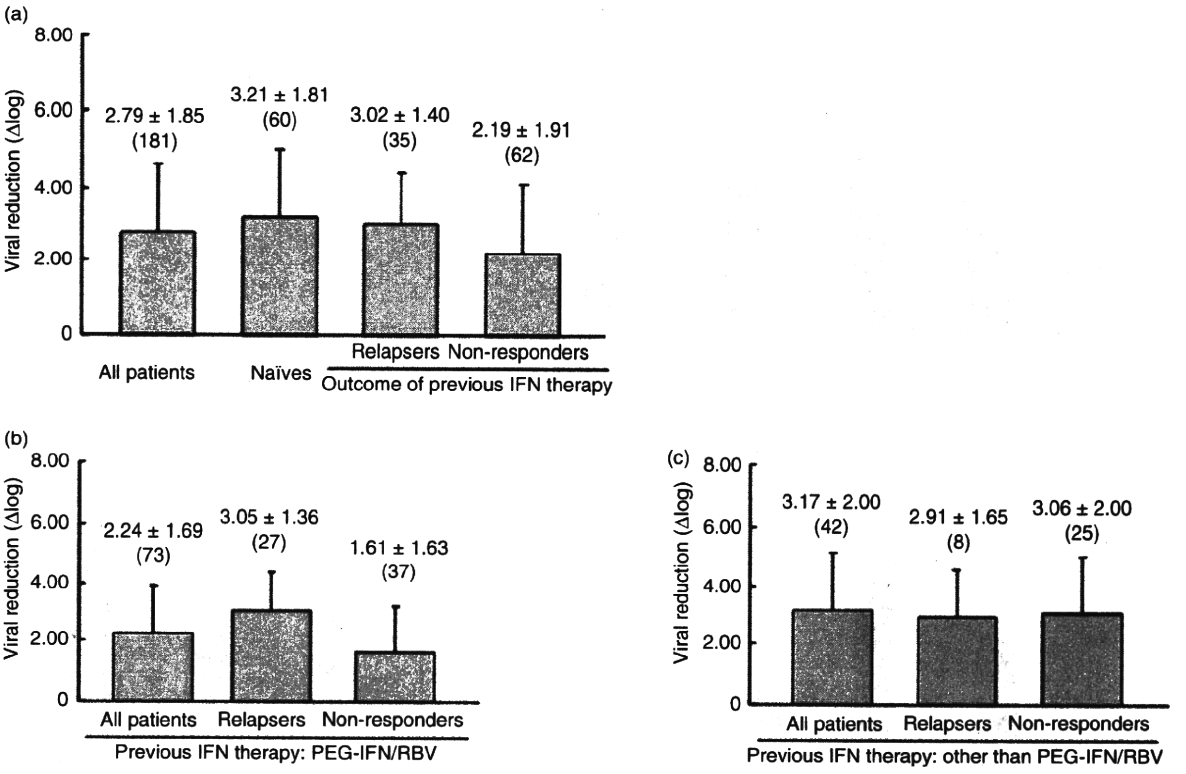

Figure 2 Viral reduction after 4 weeks treatment in patients treated with PEG IFN/RBV + DFPP. (a) All patients. (b) Previous IFN therapy: PEG IFN/RBV. (c) Previous IFN therapy: other than PEG IFN/RBV. Data are expressed as mean ± standard deviation (Δlog IU/mL). In parentheses, number of patients are given. DFPP, double filtration plasmapheresis; IFN, interferon; PEG, pegylated; RBV, ribavirin.

therapy, the reduction in viral load after 4 weeks of treatment for all patients, relapsers and non-responders was 3.17 ± 2.00, 2.91 ± 1.65 and 3.06 ± 2.00 log IU/mL, respectively (Fig. 2c). The rate of cEVR in all patients, relapsers and non-responders was 69.0% (29/42), 37.5% (3/8) and 76.0% (19/25), respectively (Fig. 3c). In PPS analysis, cEVR was 74.3% (26/35), 60.0% (3/5) and 72.7% (16/22), respectively.

Safety

Of the 239 patients evaluated for safety, 90 adverse events were observed in 55 patients (23.0%). Incidence of adverse events and puncture-site injury is presented in Table 3. Major adverse events were "minor disorder" (11 patients, 4.6%), "blood pressure lowering" (11 patients, 4.6%), "vomiting" (nine patients, 3.8%) and "nausea" (nine patients, 3.8%). Moreover, puncture-site injuries were reported in 15 patients (6.3%), mainly comprising "puncture-site infection" (seven patients, 2.9%) and "puncture-site bleeding" (four patients, 1.7%).

A total of four patients experienced adverse events judged as serious: three patients with "puncture-site infection" and one patient with "puncture-site hematoma". Bacteremia was found in one patient with "puncture-site infection" (the day after the third session of DFPP). As a result, subsequent sessions of DFPP were discontinued, however, symptoms were relieved by the 17th day after the removal of the catheter and the administration of antibiotics. In the other two patients, infection occurred during the second or the fifth session of DFPP, but DFPP was able to be continued after replacing the catheter and administrating antibiotics. The patient with "puncture-site hematoma" had been taking warfarin and was on continuous heparin therapy due to a history of atrial fibrillation. Vascular access was

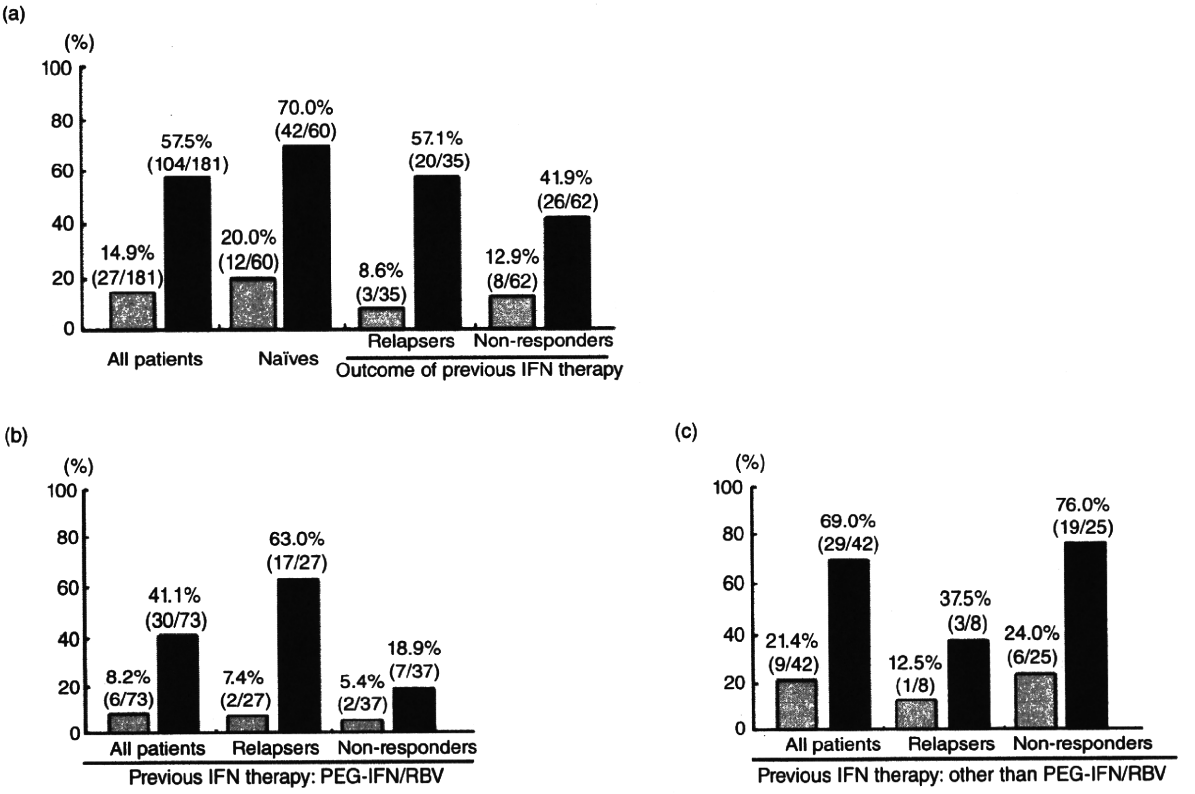

Figure 3 RVR and cEVR rates in patients treated with PEG IFN/RBV + DFPP. (a) All patients. (b) Previous IFN therapy: PEG IFN/RBV. (c) Previous IFN therapy: other than PEG IFN/RBV. In parentheses, number of patients are given. □, RVR; ■, cEVR. cEVR, complete early virological response; DFPP, double filtration plasmapheresis; IFN, interferon; PEG, pegylated; RBV, ribavirin; RVR, rapid virological response.

performed through the internal carotid artery. As dyspnea and headache were observed at the time hematoma was discovered, the physician judged the situation as "life-threatening". When the area around the hematoma was cooled and continuous heparin infusion was discontinued, the hematoma was naturally absorbed, and the patient recovered during the following day. Newly reported adverse events in this study included "epigastric discomfort" and "fibrinogen lowering" in two patients each, and "dizziness", "anorexia", "left arm numbness", "pneumonia", "cytopenia" and "thrombocytopenia" in one patient each.

This study showed that 92.9% of patients (222/239) completed five sessions of DFPP. A total of 2.9% of patients (7/239) discontinued DFPP after occurrence of an adverse event, comprising "blood pressure lowering" (first session of DFPP), "puncture-site hematoma" (second session of DFPP), "puncture-site infection", "puncture-site swelling" (third session of DFPP), and "dizziness", "thrombocytopenia" and "fibrinogen lowering" (fourth session of DFPP). In all cases, outcomes were relieved or had recovered by final follow up.

The results of factorial analysis for incidence of adverse events show higher incidence rates for women than men ($P = 0.033$). Results show that 72.7% (8/11) of "blood pressure lowering", 77.8% (7/9) of "nausea", 77.8% (7/9) of "vomiting" and 63.6% (7/11) of "minor disorder" events occurred in women. None of these events were serious, and all were relieved or recovered within a day without any treatment or with administration of physiological saline, fluid infusion or an antiemetic. No patients discontinued subsequent sessions of DFPP due to these adverse events. With the exception of sex, no other background characteristics showed any

Table 2 Factorial analysis of cEVR rate after 12 weeks of treatment

Items		*n*	No. of cEVR	cEVR rate (%)	*P*-value (Fisher's exact test)
Patients		181†	104	57.5	-
Sex	Male	90	45	50.0	0.051
	Female	91	59	64.8	
Age (years)	<60	96	53	55.2	0.541
	≥60	85	51	60.0	
Female, >50 years old	Yes	78	47	60.3	0.546
	No	103	57	55.3	
PLT ($\times 10^4/\mu L$)	<10	30	8	26.7	<0.001
	≥10	147	81	64.0	
Fibrotic stage	0–2	58	37	63.8	0.051
	3–4	17	6	35.3	
Anticoagulant	Nafamostat mesilate	115	63	54.8	1.000
	Heparin	29	16	55.2	
PEG IFN	PEG IFN-α-2a	48	30	62.5	0.496
	PEG IFN-α-2b	133	74	55.6	

†All patients treated with PEG IFN/RBV + DFPP.
cEVR, complete early virological response; DFPP, double filtration plasmapheresis; PLT, platelet count; PEG IFN, pegylated interferon.

difference in adverse events, even in patients aged 60 years or older ($P = 0.878$) (Table 4). Moreover, this study included three patients on dialysis, but no adverse events were observed in these three patients.

The changes in fibrinogen levels and platelet counts are shown in Figure 4. Fibrinogen levels and platelet counts before the second session of DFPP were significantly decreased compared to those before the start of the therapy (−36.2% and −23.3%, respectively) ($P < 0.001$), but levels remained essentially constant between the second and the fifth sessions of DFPP.

Adherence

Adherence was investigated in 181 patients treated with PEG IFN/RBV + DFPP. Adherence to PEG IFN of 80% or more and RBV of 60% or more at 4 weeks after the start of treatment was observed in 87.8% patients (159/181) and at 12 weeks in 81.2% (147/181). In patients aged 60 years or older, adherence at 4 weeks after the start of treatment was observed in 88.2% (75/85) and at 12 weeks in 80.0% (68/85). A total of two patients received three or less sessions of DFPP: one patient who underwent two sessions, and one patient with one session. In the 181 patients treated with PEG IFN/RBV + DFPP, mean volume of plasma treated per DFPP was 50.0 ± 6.5 mL/kg, thus the targeted plasma treatment volume was achieved.

DISCUSSION

THE EARLIER STUDY reported that the viral reduction after 4 weeks of treatment had been 2.43 ± 1.07 log IU/mL and SVR had been achieved in 70.8% (17/24) of patients overall.[7] In this study, PEG IFN/RBV + DFPP therapy presented comparable viral reduction of 2.79 ± 1.85 log IU/mL and cEVR rate of 57.5% (104/181) of patients. It has been reported that SVR rate is closely related to the viral dynamics in the early stage of IFN therapy,[8] even in the combination therapy of IFN and DFPP.[7] Therefore, we expect that high SVR rates would be achieved in this study, as well as the earlier report.

The results of analyses by patient background characteristics reveal that cEVR rate is significantly lower in patients with platelet counts of less than $10 \times 10^4/\mu L$. Thus, DFPP is recommended for patients with a platelet count of $10 \times 10^4/\mu L$ or more. In elderly patients aged 60 years or older and even in women aged 50 years or older, in whom IFN therapy is generally considered to be refractory,[9] no differences in cEVR were observed ($P = 0.541$, 0.546, respectively). Therefore, use of DFPP in combination with IFN therapy appears to increase SVR in elderly patients, even in women aged 50 years or older. Although the reason why no difference in cEVR was observed in elderly patients is not clear, it might be because no difference in adherence was observed

Table 3 Incidence of adverse events

Symptom	No. of patients (%)	Incidents
Gastrointestinal disorder	20 (8.4)	24
Vomiting	9 (3.8)	11
Nausea	9 (3.8)	11
Epigastric discomfort	2 (0.8)	2
General disorder	17 (7.1)	24
Minor disorder	11 (4.6)	18
Fever	2 (0.8)	2
Chills	2 (0.8)	2
Chest discomfort	2 (0.8)	2
Investigations	15 (6.3)	16
Blood pressure lowering	11 (4.6)	12
Fibrinogen lowering	2 (0.8)	2
Cytopenia	1 (0.4)	1
Thrombocytopenia	1 (0.4)	1
Nervous system disorder	4 (1.7)	4
Headache	1 (0.4)	1
Dizziness	1 (0.4)	1
Left arm numbness	1 (0.4)	1
Loss of consciousness	1 (0.4)	1
Metabolism and nutrition disorder	1 (0.4)	1
Anorexia	1 (0.4)	1
Infection and infestation	1 (0.4)	1
Pneumonia	1 (0.4)	1
Vascular disorder	1 (0.4)	1
Shock	1 (0.4)	1
Puncture-site injury	15 (6.3)	19
Infection	7 (2.9)	7
Bleeding	4 (1.7)	8
Hematoma	3 (1.3)	3
Swelling	1 (0.4)	1

whether patients were older than 60 years old or not. In this study, we could not evaluate the viral factors such as the number of mutations in IFN sensitivity determining regions (ISDR) or mutations in the RNA of core protein regions of HCV, because of the limitation of public health insurance coverage. So, we want to clarify such viral factors that influence the efficacy of this therapy in the near future.

Complete early virological response in patients with previous PEG IFN/RBV therapy occurred in 63.0% of relapsers and 18.9% of non-responders. Kaiser *et al.*[10] reported that retreatment of PEG IFN-α-2a/RBV in 107 relapsers to PEG IFN/RBV therapy achieved 43% in cEVR, and Jensen *et al.*[11] reported that retreatment of PEG IFN-α-2a/RBV in 469 non-responders to PEG IFN-α-2b/RBV achieved only 13% in cEVR. Therefore, it is expected that the addition of DFPP to PEG IFN/RBV therapy is effective in retreatment of relapsers and non-responders to previous PEG IFN/RBV therapy.

In the earlier study,[7] all retreated patients had previously been treated with other than PEG IFN/RBV, so we compared them with the 42 patients with the same history of IFN therapy in this study. The reduction in viral load after 4 weeks of treatment in the earlier study was 2.47 ± 1.11 log IU/mL, which was significantly higher than that in the non-DFPP group (1.52 ± 1.08 log IU/mL), and SVR was achieved in 77.8% of retreated patients. In this study, the reduction in viral load after 4 weeks of treatment was 3.17 ± 2.00 log IU/mL and cEVR was 69.0% in retreated patients. This cEVR is higher than the 58.8% cEVR reported by Furusyo *et al.*[12] So, we expect that the com-

Table 4 Factorial analysis of adverse events

Items		*n*	No. of patients with incidence	Incidence (%)	*P*-value (Fisher's exact test)
Patients		239	55	23.0	-
Sex	Male	113	19	16.8	0.033
	Female	126	36	28.6	
Age (years)	<60	126	30	23.8	0.878
	≥60	113	25	22.1	
PLT ($\times 10^4/\mu L$)	<10.0	41	13	31.7	0.221
	≥10.0	190	41	21.6	
Fibrotic stage	0–2	88	12	13.6	0.381
	3–4	29	6	20.7	
Anticoagulant	Nafamostat mesilate	141	32	22.7	0.381
	Heparin	40	6	15.0	
PEG IFN	PEG IFN-α-2a	67	12	17.9	0.305
	PEG IFN-α-2b	172	43	25.0	

PLT, platelet count; PEG IFN, pegylated interferon.

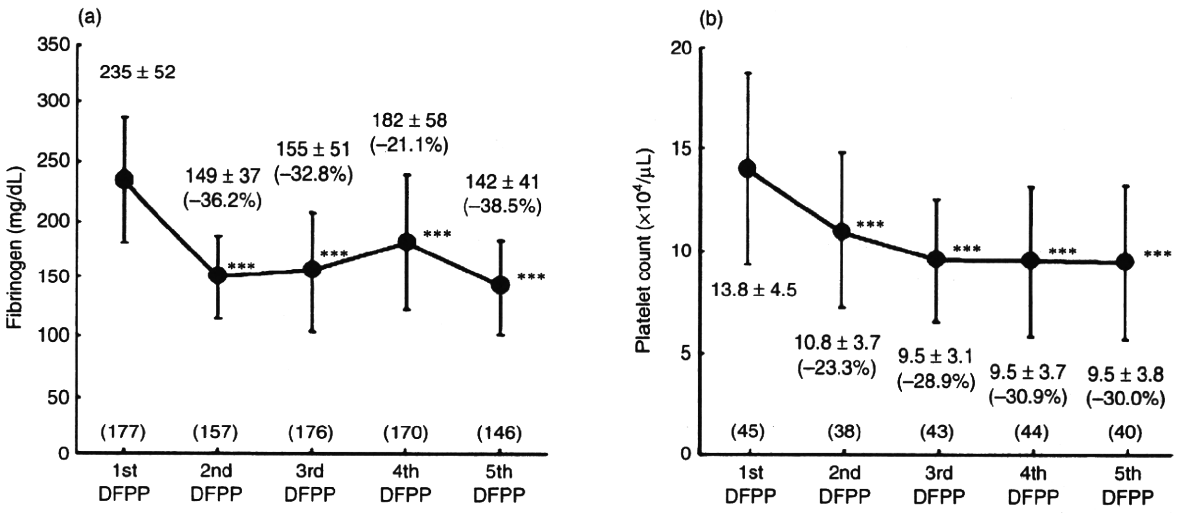

Figure 4 Changes in (a) fibrinogen and (b) platelet count during double filtration plasmapheresis (DFPP) in combination with interferon therapy. Data are expressed as mean ± standard deviation. Number of patients are given in parentheses. ***Significant value $P < 0.001$ (compared to first DFPP). Repeated-measures ANOVA and Bonferroni's correction.

bination use of IFN and DFPP is effective for retreated patients who had been previously treated other than PEG IFN/RBV therapy.

A cEVR rate of 70.0% (42/60) was attained in treatment-naïve patients through the combined use of PEG IFN/RBV and DFPP. This cEVR rate was higher than the 39.2% (74/189) obtained with PEG IFN-α-2a/RBV and 44.0% (84/191) obtained with PEG IFN-α-2b in treatment-naïve patients reported by Bisceglie *et al.*[13] Further study of the combined use of IFN therapy and DFPP on treatment-naïve patients should be conducted in larger numbers of patients.

The incidence rate for adverse events was 23.0% (55/239), lower than the 38.3% (23/60) reported in the earlier study.[7] Comparable to earlier reports, adverse events in this study appear characteristic of DFPP. Moreover, discontinuation rate of DFPP due to adverse events were low, at 2.9%, and five sessions of DFPP were performed in 92.9% of patients. These results indicate that DFPP can be safely performed in combination with IFN therapy in chronic hepatitis C patients. Puncture-site infection was reported as a serious adverse event in three patients. DFPP is normally performed after inserting a catheter, but each of these three patients had a double-lumen catheter installed in the femoral vein. Catheterization in the femoral vein is commonly performed, but must be used with caution for infection.

The results of factorial analyses by patient background characteristics indicate that adverse event rates are higher in women ($P = 0.033$), but no reports have yet indicated any sex differences in incidence rates of adverse event with DFPP, and this issue will require further study with additional numbers of patients. The fact that no differences in adverse event rates are seen in elderly patients aged 60 years or older ($P = 0.878$) suggests that this treatment can be safely performed even in the elderly.

The largest decrease in platelet count observed during DFPP treatment (~2 weeks) was 30.9%. Karino *et al.* reported a decrease of approximately 20% at 1 week after the start of IFN therapy.[14] Although platelet counts are also affected by IFN administration, variations in platelet counts during DFPP need further study.

Double filtration plasmapheresis is a therapy that was developed based on the concept that removing HCV through size separation and changing the state of HCV in the blood would enhance the therapeutic effects of IFN therapy.[4,6] DFPP has been approved in Japan for treatment of 23 diseases, such as familial hypercholesterolemia and macroglobulinemia, through the removal of disease-related lipids or proteins including immunoglobulin. Recent reports have indicated that lipids play an important role in HCV proliferation.[15–17] Hirashima *et al.*, on the other hand, reported that reduction in plasma lipids such as low-density lipoprotein cholesterol and triglycerides was observed by performing DFPP.[18] These results suggest that DFPP might affect HCV proliferation in the liver by not only removing

HCV by size separation, but also by removing lipids from the plasma. Because not all the patients enrolled in this study have hyperlipidemia, we could not measure plasma lipids with public health insurance. So, we are now planning a study to investigate whether DFPP affect plasma lipids and lipid metabolism in the liver.

Most discussion focused on patients treated with a combination of PEG IFN/RBV and DFPP in this report. However, various methods of DFPP treatment have been used, such as concomitant use of IFN-β and DFPP during PEG IFN/RBV therapy for patients with no virological response to the therapy.[19] Moreover, it has been reported that DFPP can be effective for HCV-related cryoglobulinemic glomerulonephritis,[20] and may also prevent HCV re-infection in liver transplant patients,[21] and has a dramatic effect on fibrosing cholestatic hepatitis after transplantation,[22] so other innovations using this therapy are expected.

In conclusion, this prospective study shows that the addition of DFPP to PEG IFN/RBV therapy can improve the viral reduction in the early stage of the therapy and achieve high cEVR rates in retreated and treatment-naïve patients, even in elderly patients or women aged 50 years or older, in whom IFN therapy is generally refractory. Moreover, this therapy could be safely conducted, even in the elderly. So, we consider that this therapy is useful for patients with chronic hepatitis C to prevent liver cirrhosis or cancer. We are now accumulating SVR data in this study and will subsequently conduct a control study with an ethical consideration in the near future to demonstrate the efficacy of this combination therapy more definitely.

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