Optimal amount of monocyte chemoattractant protein-1 enhances antitumor effects of suicide gene therapy against hepatocellular carcinoma by M1 macrophage activation

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Suicide gene therapy combined with chemokines provides significant antitumor efficacy. Coexpression of suicide gene and monocyte chemoattractant protein-1 (MCP-1) increases antitumor effects in murine models of hepatocellular carcinoma (HCC) and colon cancer. However, it is unclear whether the doses administered achieved the maximum antitumor effects. We evaluated antitumor effects of various amounts of recombinant adenovirus vector (rAd) expressing MCP-1 in the presence of a suicide gene in a murine model of HCC. HCC cells were transplanted subcutaneously into BALB/c nude mice, and transduced with a fixed amount of Ad-tk harboring the suicide gene, *HSV-tk*, and various doses of Ad-MCP1 harboring MCP-1 (ratios of 1:1, 0.1:1, and 0.01:1 relative to Ad-tk). Growth of primary tumors was suppressed when treated with Ad-tk plus Ad-MCP1 (1:1 and 1:0.1) as compared with Ad-tk alone. The antitumor effects against tumor rechallenge tended to be high in the Ad-tk plus Ad-MCP1 group (1:0.1). The effects were dependent on production of Th1 type-cytokines. Delivery of an optimal amount of rAd expressing MCP-1 enhanced the antitumor effects of suicide gene therapy against HCC by M1 macrophage activation, suggesting that this is a plausible form of cancer gene therapy to prevent HCC progression and recurrence. (*Cancer Sci* 2008; 99: 2075–2082)

Cancer gene therapy using combinations of various genes, such as suicide and cytokine genes, to enhance tumor regression therapy is widely used.[1,2] Previously, we reported that the coexpression of herpes simplex virus thymidine kinase (HSV-tk) and monocyte chemoattractant protein-1 (MCP-1) showed enhanced antitumor effects in models of hepatocellular carcinoma (HCC)[3] and colon cancer,[4] and these antitumor effects were dependent on the activation of macrophages.[3] 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,[5] and is functional in both mice and humans.[6] However, MCP-1 was reported to be destructive in some tumor models,[6,7] but protective in others.[8] Monocytes/macrophages recruited by MCP-1 have dual functions in that they can prevent the establishment and spread of tumor cells,[6] and simultaneously support tumor growth and dissemination.[8] This ambivalent relationship reflects the elevated functional plasticity of macrophages, which are able to express different functional programs in response to different microenvironment signals, as exemplified in the M1 (classical)–M2 (alternative or non-classical) paradigm of macrophage polarization.[9]

On the other hand, although double infection methods are used to enhance antitumor effects in cancer gene therapy, significant antitumor effects have been reported in some studies,[4,10] but not in others.[11,12] Moreover, it is not clear how the antitumor effects are affected by differences in the doses administered. In the present study, various amounts of recombinant adenovirus vector (rAd) expressing the *MCP-1* gene were delivered into cells along with the same amount of HSV-tk to determine the optimal dosage of MCP-1 for induction of stronger antitumor effects in double infection methods. Furthermore, we also examined the involvement of macrophage immune responses in these effects. Here, we demonstrated that treatment with the 1:0.1 ratio of Ad-HSV-tk (Ad-tk) plus Ad-MCP1 tended to exert antitumor immunity, suggesting that there may be an optimal amount of Ad-MCP1 in suicide gene therapy. In addition, it is possible that the antitumor responses seen in the HSV-tk plus MCP-1 system were associated with increased Th1 (T helper 1)–type cytokine production by activated M1 macrophage. These findings will be of value in cancer gene therapy.

Materials and Methods

Recombinant adenoviruses. rAds harboring the human MCP-1 (Ad-MCP1), HSV-tk (Ad-tk), and lacZ (Ad-lacZ), and driven by the CAG promoter were prepared, purified, and titrated according to the protocols supplied by the manufacturer (Takara Bio, Shiga, Japan), as described.[13] The rAds were purified on cesium gradients and their titers were determined by the 50% tissue culture infectious dose ($TCID_{50}$).

Cell lines and culture. The human HCC cell line Huh7 and the mouse HCC cell line BNL 1ME A.7R.1 (BNL) were cultured in Dulbecco's minimal essential medium (Gibco, Long Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco).

Enzyme-linked immunosorbent assay (ELISA) for MCP-1. Aliquots of 1×10^5 Huh7 cells were seeded in 1.0 mL of culture media in 24-well tissue culture plates. Twenty-four h later, the cells were infected with each rAd at a multiplicity of infection (MOI) of 10, and the medium was collected 48 h later. On the other hand, in some experiments, ganciclovir (GCV; Tanabe Pharmaceutical Drug, Tokyo, Japan) (10 µg/mL) was added 72 h later, and the medium was collected and replaced with the same volume of fresh medium every 24 h. The concentration of MCP-1 in the medium collected from each well was determined by ELISA as described.[14]

***In vivo* studies in nude mice.** The following investigations were performed in accordance with the guidelines of our Institutional Animal Care and Use Committee. Six-week-old male athymic

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nude mice (BALB/cA Jcl-nu; CLEA Japan, Tokyo, Japan) were injected subcutaneously with 1×10^7 HuH7 cells at the both sides of the flank on day 0. On days 3 and 4, 1×10^7 $TCID_{50}$ (100 µL) of Ad-tk, Ad-lacZ, or Ad-tk (1×10^7 $TCID_{50}$, fixed dose) plus Ad-MCP1 (1, 0.1, 0.01, or 0.001×10^7 $TCID_{50}$, changed dose) were injected into the tumor. Then, 75 mg/kg of GCV was administered into the peritoneal cavity daily for the next 5 consecutive days (day 5–9), and tumor size was measured every 3 days. Tumor volumes were calculated using the formula:

$$\frac{(\text{longest diameter}) \times (\text{shortest diameter})^2}{2}$$

Gene expression analysis (real-time reverse transcription–polymerase chain reaction [RT-PCR]). Total RNA was extracted from tumor tissues or spleens resected after treatment of the tumor with each rAd, using a Total Cellular RNA Isolation Kit (Ambion, St. Austin, TX, USA), in accordance with the manufacturer's protocol. The RNA was reverse transcribed with a TaqMan reverse transcription reagent kit (PE Applied Biosystems, Foster City, CA, USA) using random hexamer primers. Gene expression was analyzed by real-time RT-PCR using TaqMan Universal Master Mix on an ABI PRISM 7900 Sequence Detection System (PE Applied Biosystems). The PCR primer pairs for mouse interleukin (IL)–10, IL-12, IL-18, IFN-γ, VEGF, and 18S rRNA were obtained from the TaqMan assay reagent library. Data for whole samples were normalized to 18S rRNA and then expressed as the fold change in mRNA expression as compared with control samples treated with phosphate-buffered saline (PBS).

Immunohistochemical analysis. Tumor tissues were resected on day 10. The tissue samples were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Cryostat sections of frozen tissues were fixed in cold acetone for 10 min, followed by three rinses in PBS. To avoid non-specific staining, avidin and biotin in the tissues were blocked using a blocking kit (Vector Laboratories, Burlingame, CA, USA). The slides were subsequently incubated with antibodies (Abs) against Mac-1 (M1/70; Pharmingen, San Diego, CA, USA) for 30 min at room temperature. Negative controls included staining with non-specific Ab of the corresponding isotype and subsequent staining with secondary Ab. The reactions were visualized using a VECTASTAIN ABC Standard Kit (Vector Laboratories), followed by counterstaining with hematoxylin.

Preparation of peritoneal exudate macrophages and assays for cytokine production *in vitro*. Thioglycolate-elicited murine peritoneal exudate cells were collected as described.[15] Briefly, nude or immunocompetent mice were injected intraperitoneally with 2 mL each of 3% fluid thioglycolate medium (Wako Pure Chemical) and sacrificed 4 days later, followed by peritoneal lavage with 10 mL of cold PBS. About 90% of the collected peritoneal cells were positive for both Mac-1 (CD11b) and $I\text{-}A^d$ MHC class II as determined by staining with PE-conjugated anti-Mac-1 Ab and fluorescein-isothiocyanate (FITC)–conjugated $I\text{-}A^d$ MHC class II (AMS-32.1; Pharmingen). Huh7 cells were infected with rAds, at a MOI of 5 for 24 h. Aliquots of 10^5 macrophages were cocultured with 10^5 rAd-treated Huh7 cells in 1.0 mL of culture media in 24-well tissue culture plates, and treated with GCV for 2 days at 37°C. The concentrations of IL-10, IL-12, IL-18, and IFN-γ in the media were quantified using immunoassay kits (IL-10, IL-12, IFN-γ: Biosource International, Camarillo, CA, USA; IL-18: Medical & Biological Laboratories, Nagoya, Japan).

Rechallenge testing in nude mice. Nude mice were injected subcutaneously with 5×10^6 HuH7 cells on day 0. On days 3 and 4, the subcutaneous tumors were injected with 5×10^7 $TCID_{50}$ (100 µL) of Ad-tk, Ad-lacZ, or Ad-tk (fixed dose) plus Ad-MCP1 (changed dose), and the mice were treated with 75 mg/kg GCV, injected into the peritoneal cavity, every day for the next 5 days (days 5–9). Following complete eradication of the primary tumors, the mice were subcutaneously rechallenged on day 14 with 3×10^6 HuH7 cells at two sites, which were more than 3 cm apart from the primary challenge site. Two of 10 (20%) mice treated with Ad-tk and four of 30 (13.3%) treated with Ad-tk plus Ad-MCP1 did not show complete eradication of the primary tumor by the final measurement and were therefore excluded from the rechallenge experiment. Tumor sizes were measured every 4 days after the second tumor injection, and tumor volumes were calculated using the formula:

$$\frac{(\text{longest diameter}) \times (\text{shortest diameter})^2}{2}$$

Animal studies in immunocompetent mice (*ex vivo*, *in vivo*, and rechallenge). Six-week-old immunocompetent male BALB/c-jcl mice (CLEA Japan) were injected subcutaneously with 1×10^5 BNL cells infected with each rAd at an *in vitro* MOI of 5 at the both sides of the flank on day 0, and GCV was administered intraperitoneally for the next 5 days (days 1–5). Tumor size was measured every 7 days, and tumor volume was calculated using the formula:

$$\frac{(\text{longest diameter}) \times (\text{shortest diameter})^2}{2}$$

As with the experiments on nude mice, BALB/c-jcl mice were injected subcutaneously with 1×10^5 BNL cells at the both sides of the flank on day 0. On days 3 and 4, 5×10^5 $TCID_{50}$ (100 µL) of rAds were injected into the tumor. Then, GCV was administered for the next 5 days (day 5–9), and tumor size was measured every 3 days.

In another experiment, immunocompetent mice were injected subcutaneously with 1×10^5 BNL cells infected with each rAd at an *in vitro* MOI of 100 on day 0, and GCV was administered intraperitoneally for the next 5 days (days 1–5). The primary tumors were completely eradicated in all groups. These mice were injected subcutaneously with 1×10^4 BNL cells on day 14 at two sites which were separate from the primary challenge sites, and the tumor sizes were measured every 7 days after the second tumor injection.

ELISA for serum IL-10, IL-12, and IL-18. Mouse sera were collected prior to injection of subcutaneous primary tumors and on day 35 after tumor injection. IL-10, IL-12, and IL-18 concentrations were measured using immunoassay kits (IL-12, Biosource International; IL-18, Medical & Biological Laboratories).

Flow cytometry. Single-cell suspensions of splenocytes were resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide, and incubated for 30 min on ice with FITC-conjugated rat antimouse-F4/80 (Serotec, Oxford, UK) and PE-conjugated rat antimouse pan natural killer (NK) cells (DX5; Pharmingen), with FITC-conjugated rat antimouse-CD3 (Pharmingen) and PE-conjugated rat antimouse CD11c (Pharmingen) or with FITC-conjugated rat antimouse-CD8 (Pharmingen) and PE-conjugated rat antimouse CD4 (Pharmingen). The cells were washed, resuspended in PBS, and analyzed using a FACScan with CellQuest software.

Statistical analysis. All results are expressed as means ± SE. The statistical significance of differences between groups was evaluated by the Mann–Whitney *U*-test.

Results

MCP-1 production by double infection with recombinant adenoviruses *in vitro*. MCP-1 expression level by Ad-MCP1 alone was high compared with double infection of Ad-lacZ plus Ad-MCP1 (Fig. 1a). The amounts of MCP-1 produced by Ad-tk plus Ad-MCP1 decreased rapidly after GCV administration due to Huh7 cell

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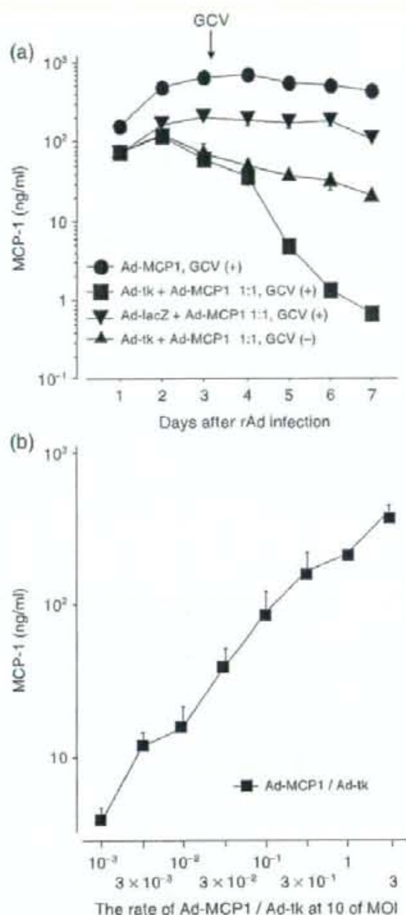

Fig. 1. Monocyte chemoattractant protein-1 (MCP-1) production of recombinant adenoviruses in the presence of herpes simplex virus thymidine kinase (HSV-tk). Aliquots of 1×10^5 Huh7 cells were seeded in 1.0 mL of culture media in 24-well tissue culture plates. (a) Twenty-four h later, the cells were treated with Ad-tk plus Ad-MCP1, Ad-lacZ plus Ad-MCP1, or Ad-MCP1 at a multiplicity of infection (MOI) of 10, and treated 72 h later with ganciclovir (GCV) (10 μg/mL). Every 24 h, the medium was collected and replaced with the same volume of fresh medium. (b) Twenty-four h later, the cells were doubly infected with Ad-tk (fixed dose, at an MOI of 10) plus Ad-MCP1 (changed dose), and the medium was collected 48 h later. The concentrations of MCP-1 were evaluated using an immunoassay. Values are shown as the means ± SE of duplicate experiments.

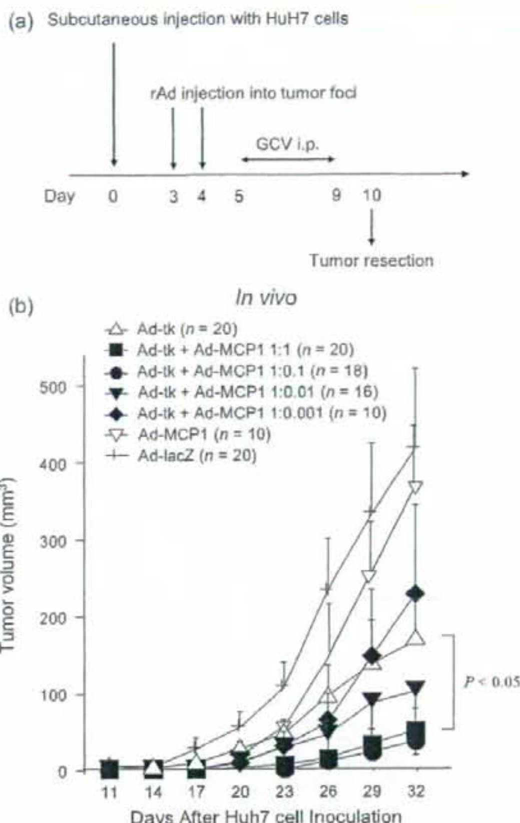

Fig. 2. The antitumor effects of the herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir (GCV) system by codelivery of monocyte chemoattractant protein-1 (MCP-1) in a nude mouse model of hepatocellular carcinoma (HCC). (a) Mice were injected subcutaneously with 1×10^7 Huh7 cells at the both sides of the flank on day 0. On days 3 and 4, 1×10^7 $TCID_{50}$ of Ad-tk, Ad-tk (1×10^7 $TCID_{50}$, fixed dose) plus Ad-MCP1 (1, 0.1, 0.01, or 0.001×10^7 $TCID_{50}$, changed dose), or Ad-lacZ was injected into the tumor, and the mice were injected intraperitoneally (i.p.) with 75 mg/kg of GCV every day for the next 5 days (day 5–9). (b) Tumor size was measured every 3 days. The results are shown as the means of two independent experiments.

apoptosis induced by the HSV-tk/GCV system (Fig. 1a). Moreover, the amounts of MCP-1 produced by Ad-tk plus Ad-MCP1 without GCV administration were lower than those of Ad-lacZ plus Ad-MCP1, presumably due to the MCP-1 promoter interference by HSV-tk.

Next, production of MCP-1 in Huh7 cells double-infected with Ad-tk (fixed dose) plus Ad-MCP1 (changed dose) was measured. The amounts of MCP-1 were correlated with the infectious dose of Ad-MCP1 in the presence of a fixed amount of HSV-tk (Fig. 1b).

Antitumor effects of the HSV-tk/GCV system by codelivery of the *MCP-1* gene in an athymic nude mouse model of HCC. The *in vivo* antitumor effects of double infection with rAds were analyzed using athymic nude mice (Fig. 2a). The growth of subcutaneous tumors was markedly suppressed in animals treated with Ad-tk plus Ad-MCP1 (1:1) (tumor volume 32 days after injection, 44.4 ± 22.5 mm^3) or Ad-tk plus Ad-MCP1 (1:0.1) (37.4 ± 18.6 mm^3), as compared to those treated with Ad-tk alone (170.2 ± 49.8 mm^3, $P < 0.05$) (Fig. 2b). These observations indicated that optimal amounts of MCP-1 are needed to eradicate tumor cells in the presence of HSV-tk.

Recruitment and activation of macrophages into tumor tissues. Macrophages play important roles in both Th1- and Th2-mediated immune responses. Classical macrophage (M1 macrophages) are also a major source of IL-12 and IL-18, whereas alternative macrophages (M2 macrophages) are a source of IL-10.[9] IL-12

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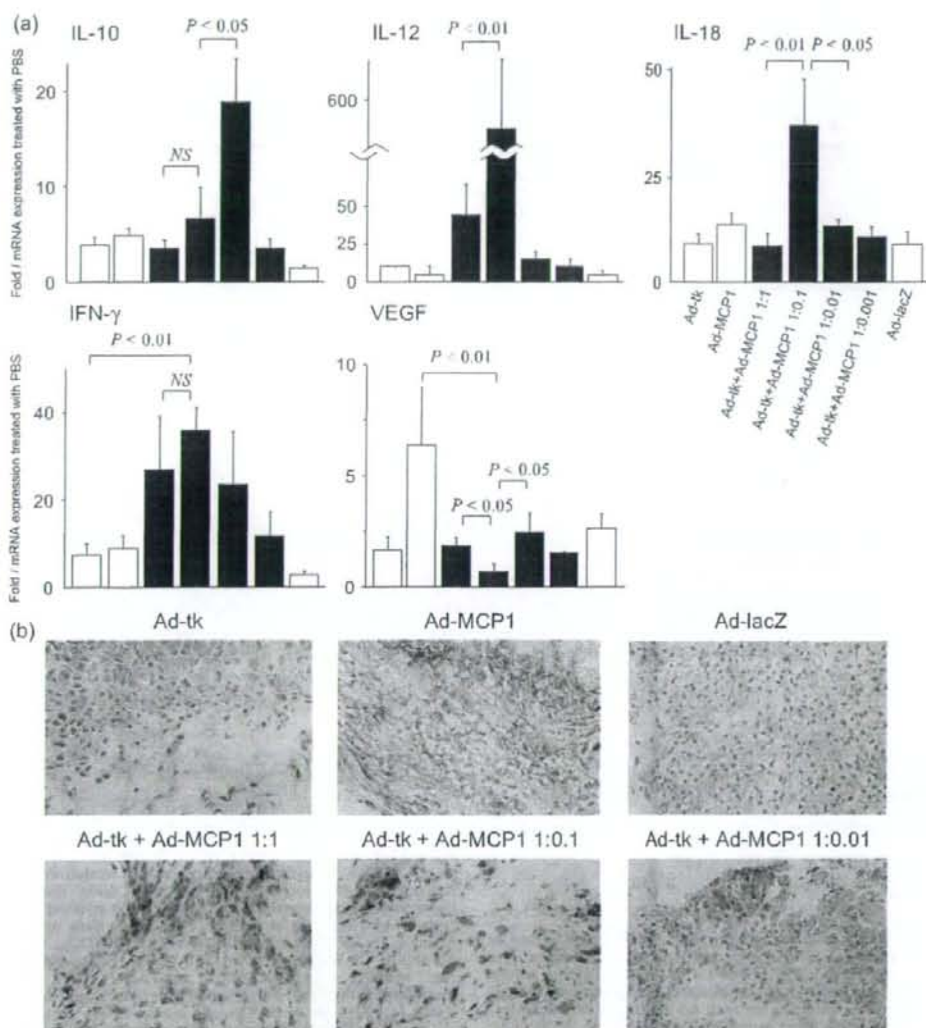

Fig. 3. Cytokine expression and macrophage recruitment in primary tumor tissues. In the experiment described in the legend to Fig. 2, tumor tissues were resected 10 days after tumor injection. (a) Total RNA was extracted to determine cytokine mRNA levels by a real-time reverse transcription–polymerase chain reaction as described in 'Materials and Methods'. Cytokine mRNA expression was normalized to 18S rRNA and then expressed as the fold change in mRNA expression as compared with control samples treated with phosphate-buffered saline. Splenocytes treated with 0.1 μg/mL LPS were used as a positive control (data not shown). The results are shown as the means of two independent experiments. (b) Tumor tissues were processed for immunohistochemical analysis using anti-Mac1 antibody as described in 'Materials and Methods'. Representative results from individual animals in each group are shown here.

enhances the activities of NK cells and cytotoxic T lymphocytes (CTL), and plays a key role in the induction of Th1-type immune responses.[16] In addition, IL-18 is a proinflammatory cytokine produced by activated macrophages, which has been shown to induce Th1 cell development and NK cell activation in combination with IL-12.[17] In contrast, the effects of IL-10 on immune responses are mostly inhibitory.[18] Therefore, to evaluate whether M1 macrophages recruited into tumor tissues following infection with rAds were activated, IL-10, IL-12, IL-18, IFN-γ, and VEGF expression were determined using real-time RT-PCR. IL-12 and IL-18 mRNA levels were significantly increased ($P < 0.01$), and that of IFN-γ mRNA tended to increase in tumors treated with Ad-tk plus Ad-MCP1 (1:0.1) (Fig. 3a). In contrast, IL-10 mRNA was significantly increased in tumors treated with Ad-tk plus Ad-MCP1 (1:0.01) ($P < 0.05$) (Fig. 3a). In addition, the VEGF mRNA level was significantly increased in tumors treated with Ad-MCP1 ($P < 0.01$), and was significantly decreased in tumors treated with Ad-tk plus Ad-MCP1 (1:0.1)

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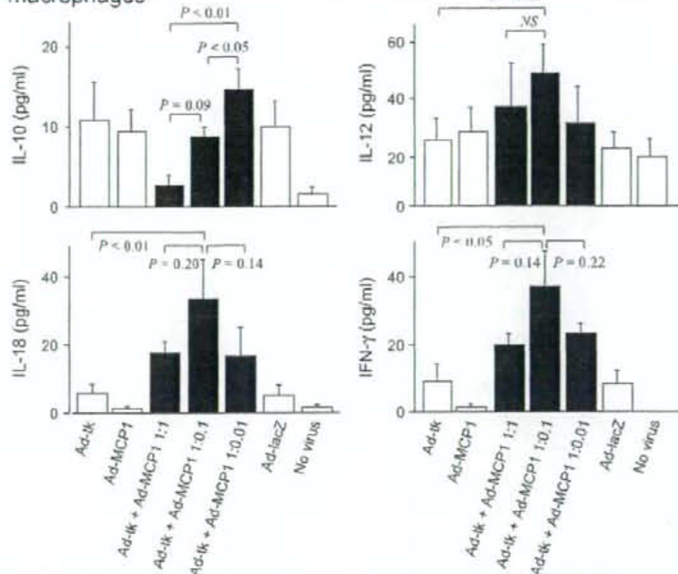

Fig. 4. Cytokine production by peritoneal macrophages cocultured with Huh7 cells infected with rAds *in vitro*. Huh7 cells were infected with each rAd at a multiplicity of infection (MOI) of 5 and treated with ganciclovir (GCV) for 24 h. Aliquots of 10^5 peritoneal exudate macrophages were cocultured with 10^5 rAd-treated Huh7 cells for 2 days, and the concentrations of IL-10, IL-12, IL-18, and IFN-γ in the media were evaluated by immunoassay. Values are shown as the means ± SE of duplicate experiments.

($P < 0.05$) (Fig. 3a). Taken together, these observations indicated that M1 macrophages were highly activated when tumors were treated with the optimal dose of MCP-1 and HSV-tk.

Next, we evaluated whether there were differences in the number of macrophages recruited into tumor tissues. The number of accumulated Mac-1-positive cells in the tumors treated with Ad-tk plus Ad-MCP1 (1:0.1) was comparable to that in those treated with Ad-tk plus Ad-MCP1 (1:1) (Fig. 3b). These observations suggested that the number of recruited macrophages is of little importance to the antitumor effects.

IL-10, IL-12, IL-18, and IFN-γ production by coculture of apoptotic Huh7 cells expressing MCP-1 and peritoneal macrophages *in vitro*. It was reported that adenoviral-mediated overexpression of MCP-1 differentially modulated the development of Th1 and Th2-type responses.[19] To evaluate the differences in the immunomodulatory effects of macrophages among double infection of rAds, we measured IL-10, IL-12, IL-18, and IFN-γ production by peritoneal exudate cells consisting mostly of macrophages, when they were cocultured with Huh7 cells infected with rAds. We found that peritoneal macrophages cocultured with Huh7 cells treated with Ad-tk plus Ad-MCP1 (1:0.1) tended to produce increased levels of IL-12, IL-18, and IFN-γ (Fig. 4). On the other hand, the increase in amount of IL-10 in the double infection groups was inversely proportional to the dosage of MCP-1 vector (Fig. 4). These observations also suggest that the optimal dose of MCP-1 and HSV-tk may induce M1 macrophage activation.

Antitumor immunity in the rechallenge test of the HSV-tk/GCV system by codelivery of the *MCP-1* gene. After primary subcutaneous Huh7 cells were completely eradicated with rAds, nude mice were rechallenged with Huh7 cells to evaluate antitumor immunity induced by MCP-1 plus HSV-tk. We found that the tumor regrowth was significantly suppressed when the primary tumor cells had been eradicated with Ad-tk plus Ad-MCP1 (1:0.1) as compared with Ad-tk (tumor volume 40 days after rechallenge: 123.2 ± 77.2 mm^3 *vs* 544.5 ± 161.6 mm^3, respectively, $P < 0.05$) (Fig. 5). In addition, tumor regrowth tended to be low when eradicated with Ad-tk plus Ad-MCP1 (1:0.1) as compared with Ad-tk plus Ad-MCP1 (1:1) (287.9 ± 137.1 mm^3, $P = 0.18$) or Ad-tk plus Ad-MCP1 (1:0.01) (269.7 ± 91.1 mm^3, $P = 0.24$). Next, to evaluate immunomodulatory effects of splenocytes, we examined IFN-γ expression using real-time RT-PCR. IFN-γ mRNA levels were significantly increased in the spleens of nude mice treated with Ad-tk plus Ad-MCP1 (1:0.1) (Fig. 5b). Consistent with our previous findings,[20] we observed increased numbers of NK cells in the spleen and rechallenged tumor tissues when treated with the 1:0.1 ratio of Ad-tk and Ad-MCP1 (data not shown). These results indicated that the optimal dose of MCP-1 induced beneficial antitumor immunity in the presence of HSV-tk.

Antitumor effects and immunity of the HSV-tk/GCV system plus MCP-1 treatment in an immunocompetent mouse model of HCC. There is no CTL in athymic nude mice. Therefore, to evaluate the Th1 cytokine response in the syngeneic system, the *ex vivo* antitumor effects of double infection with rAds were analyzed using immunocompetent BALB/c-jcl mice. The growth of subcutaneous tumors treated with Ad-tk plus Ad-MCP1 (1:1, 1:0.1) was comparable to that in nude mice ($P < 0.01$), excluding the group in which the dose of MCP-1 was small (1:0.01) (Fig. 6a).

In the next experiment, after the BALB/c mice developed tumor mass following the injection with non-infected BNL cells, we infected the resultant tumors with Ad-tk plus Ad-MCP1 and treated the animals with GCV using the same procedures as the experiments with nude mice. Tumor growth was apparently retarded when treated with Ad-tk plus Ad-MCP1 (1:1) ($P < 0.05$) and (1:0.1) ($P < 0.01$) as compared with Ad-tk alone (Fig. 6b). However, the treatments failed to eradicate tumors completely, probably because the infection efficiency was not sufficient under these conditions.

Thus, we chose the *ex vivo* infection experiment in the immunocompetent mouse model, to evaluate whether rechallenged tumors could be rejected in the mice in which the primary tumors had been completely eradicated. The immunocompetent mice were rechallenged with BNL 1ME A.7R.1 (BNL) cells

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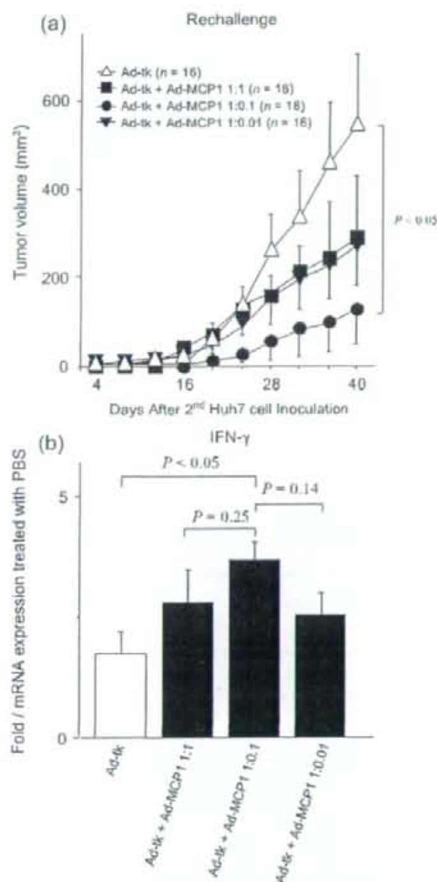

Fig. 5. Nude mice were injected subcutaneously with 5×10^6 Huh7 cells on day 0. On days 3 and 4, 5×10^7 $TCID_{50}$ of Ad-tk (100 µL), Ad-tk (1×10^7 $TCID_{50}$, fixed dose) plus Ad-MCP1 (1, 0.1, 0.01, or 0.001×10^7 $TCID_{50}$, changed dose), or Ad-lacZ was injected into the tumor, and the mice were injected intraperitoneally with 75 mg/kg of ganciclovir (GCV) every day for the next 5 days (day 5–9). Following complete eradication of the primary tumors, the mice were subcutaneously rechallenged on day 14 with 3×10^6 Huh7 cells at the other sites. (a) Tumor size was measured every 4 days. (b) In another series of experiments, the spleen was resected on day 16 after tumor injection, and IFN-γ, mRNA levels were evaluated using real-time reverse transcription–polymerase chain reaction. The results are shown as the means of two independent experiments. PBS, phosphate-buffered saline.

using the same procedures as in the experiments with nude mice. Although the inhibition of tumor regrowth was significantly lower when they had been eradicated with Ad-tk plus Ad-MCP1 (1:0.1) as compared with Ad-tk (tumor volume 42 days after rechallenge: 263.9 ± 87.8 mm³ *vs* 669.5 ± 158.3 mm³, respectively, $P < 0.05$), it also tended to be lower when the primary tumor cells had been eradicated with Ad-tk plus Ad-MCP1 (1:1) (tumor volume 42 days after rechallenge: 372.5 ± 157.8 mm³) (Fig. 6c), similar to the observations in athymic nude mice.

Next, we examined IL-10, IL-12, and IL-18 production on day 35 after tumor injection. Serum concentrations of IL-12 and IL-18 tended to be higher in mice treated with Ad-tk plus Ad-MCP1 (1:0.1) as compared with those treated with Ad-tk, Ad-tk plus Ad-MCP1 (1:1), or Ad-tk plus Ad-MCP1 (1:0.01) (Fig. 6d). In contrast, the increase in amount of serum IL-10 in the double infection groups was inversely proportional to the dosage of MCP-1 (Fig. 6d). These observations were consistent with the data shown in Figs 3 and 4.

Finally, to monitor the activation state of innate and acquired immunity in extrahepatic lymphoid organs, we examined the numbers of immune cells in the spleen on day 35 after tumor injection by FACS analysis. The numbers of F4/80-positive cells tended to be higher in the Ad-tk plus Ad-MCP1 (1:1) and Ad-tk plus Ad-MCP1 (1:0.1) groups, and the numbers of DX5-positive cells tended to be higher in the Ad-tk plus Ad-MCP1 (1:0.1) group (Fig. 6e). Furthermore, the numbers of CD3-, CD4-, and CD8-positive cells were increased in the immunocompetent mice in the order of Ad-tk plus Ad-MCP1 (1:0.1), Ad-tk plus Ad-MCP1 (1:1), and Ad-tk plus Ad-MCP1 (1:0.01) (Fig. 6e). Taken together, these results confirmed that treatment with Ad-tk plus Ad-MCP1 (1:0.1) resulted in the development of beneficial antitumor immunity in both immunodeficient and immunocompetent animals.

Discussion

HCC is one of the most common cancer-related causes of death, and is resistant to anticancer drugs.[21] Although gene therapy has the potential to more effectively induce tumor cell death as compared to conventional treatment, there have been no previous comparisons with regard to the optimal doses of vectors in combined gene therapy. Whereas the amounts of MCP-1 were correlated with the infectious dose of Ad-MCP1 in the presence of a fixed dose of Ad-tk, MCP-1 expression level in the presence of intracellular HSV-tk was inhibited as compared with coinfection with Ad-MCP1 plus Ad-lacZ, suggesting that HSV-tk may influence the efficiency of transcription in the transformed cells. In addition, MCP-1 expression level by Ad-MCP1 alone was high as compared with double infection with Ad-MCP1 plus Ad-lacZ, which was probably due to promoter interference. On the other hand, our previous study demonstrated that the levels of HSV-tk expression in cells cotransfected with Ad-tk plus Ad-MCP-1 were comparable to those of Ad-tk alone or Ad-tk plus Ad-lacZ.[3] The effect of a bicistronic rAd expressing mainly HSV-tk was clearly stronger than that of a bicistronic rAd expressing mainly MCP-1. Therefore, we proposed that the

Fig. 6. Antitumor effects of the herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir (GCV) system by codelivery of monocyte chemoattractant protein-1 (MCP-1) in an immunocompetent mouse model of HCC. (a) Mice were injected subcutaneously with 1×10^5 BNL cells infected with each rAd at an *in vitro* multiplicity of infection (MOI) of 5 at the both sides of the flank on day 0. GCV was administered intraperitoneally for the next 5 days (days 1–5), and tumor size was measured every 7 days. (b) BALB/c-jcl mice were injected subcutaneously with 1×10^5 BNL cells at the both sides of the flank on day 0. On days 3 and 4, 5×10^5 $TCID_{50}$ (100 µL) of rAds were injected into the tumor. Then, GCV was administered for the next 5 days (day 5–9), and tumor size was measured every 3 days. (c) BALB/c-jcl mice were injected subcutaneously with 1×10^5 BNL cells infected with each rAd at an *in vitro* MOI of 100 on day 0, and GCV was administered intraperitoneally for the next 5 days (days 1–5). The primary tumors were completely eradicated in all groups. These mice were injected subcutaneously with 1×10^4 BNL cells at other sites on day 14, and the tumor sizes were measured every 7 days after the second tumor injection. (d) Mouse sera were collected prior to subcutaneous injection of primary tumor cells (untreated), after treatment of the tumor with each rAd, and 2 days after rechallenge with HuH7 cells, and IL-12 and IL-18 concentrations were measured using immunoassay kits. (e) The spleen was removed to obtain single cell suspensions on day 35 after tumor injection. Surface expression of DX5, F4/80, CD3, CD4, CD8, and CD11c in cell populations obtained from the spleen were assessed by FACS. The results are representative of two independent experiments.

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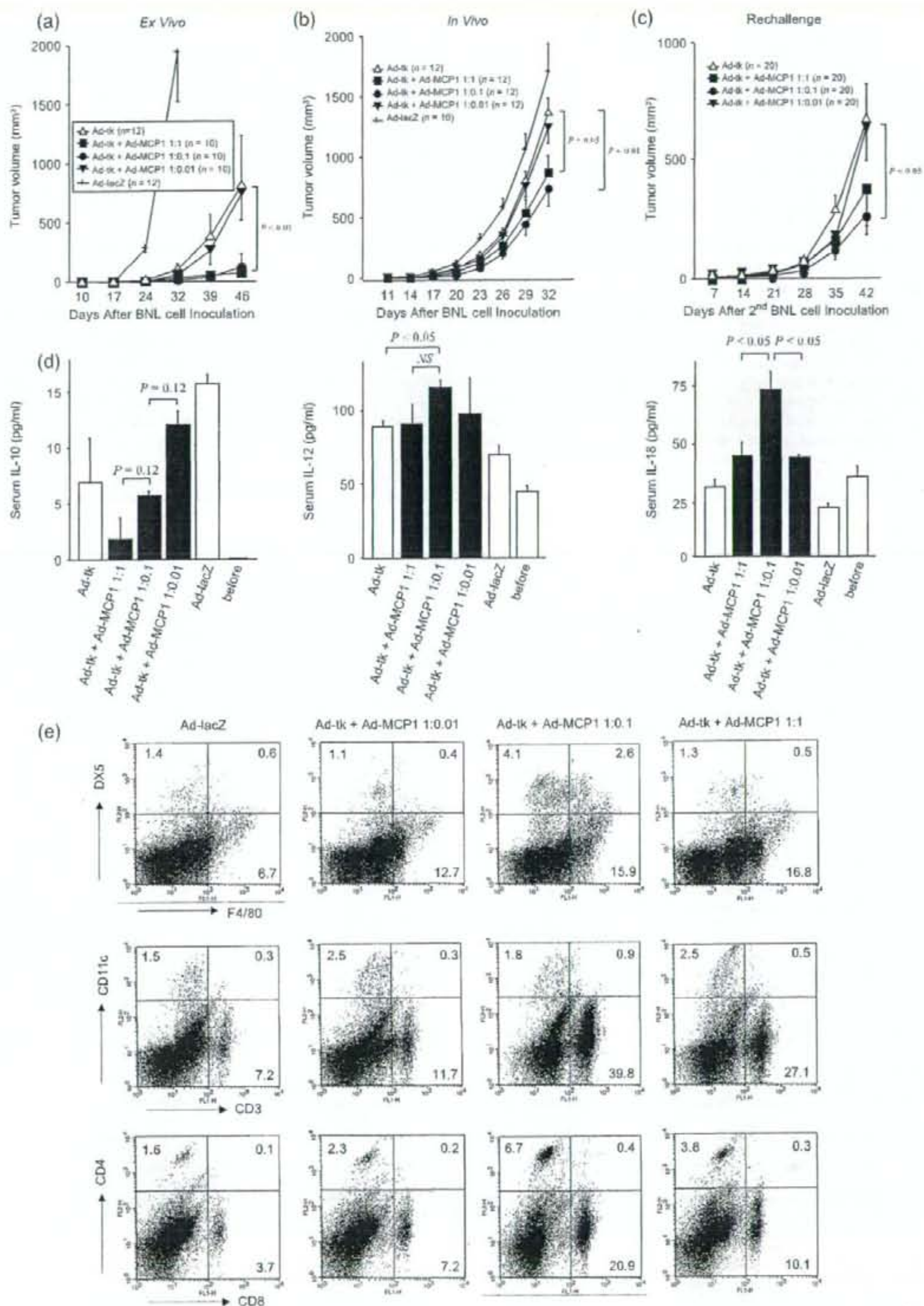

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HSV-tk/GCV system should mainly be used and the use of MCP-1 was supported in our experimental models, although their efficiencies may vary depending on the nature of the cell type and reporter genes used.[22]

Th1 cytokine expression levels in tumors treated with Ad-tk plus Ad-MCP1 (1:0.1) were higher than those treated with Ad-tk plus Ad-MCP1 (1:1) or (1:0.01). Moreover, macrophages produced large amounts of Th1 cytokines when cocultured with apoptotic HCC cells induced by Ad-tk plus Ad-MCP1 (1:0.1). In contrast, whereas the amounts of Th2 cytokines were relatively high in Ad-tk plus Ad-MCP1 (1:0.01), they were low in Ad-tk plus Ad-MCP1 (1:1). There were almost no differences in the number of macrophages among the tumors treated with various combinations of HSV-tk and MCP-1. Therefore, the types of activated macrophages may be important rather than the numbers recruited and activated. The ratio of IL-12 to IL-10 can be used as a simple metric to classify activated macrophages into two categories, M1 or M2.[23,24] M1 macrophages are potent effector cells that kill microorganisms and tumor cells and produce large amounts of proinflammatory cytokines, particularly IL-12. In contrast, M2 macrophages, a producer of IL-10, tune inflammatory responses and adaptive Th1 immunity, scavenge debris, and promote angiogenesis, tissue remodeling, and repair. The M1/M2 dichotomy of macrophage polarization can elicit both anti- and pro-tumoral activities.[25]

MCP-1 is known to facilitate tumor growth under different conditions, probably by promoting angiogenesis.[8] In the present study, the VEGF expression levels in tumors treated with Ad-tk plus Ad-MCP1 (1:0.1) were low as compared with those treated with Ad-MCP1 alone or Ad-tk plus Ad-MCP1 (1:1 and 1:0.01). A previous study indicated that monocyte recruitment is dependent on the level of MCP-1 secreted by the tumor cells and that the effects of monocyte infiltration on tumor growth are dependent on their levels of infiltration.[26] MCP-1 secreted by apoptotic Huh7 cells may have recruited macrophages more efficiently to these apoptotic cells, thereby resulting in a greater deleterious effect on tumor formation. Therefore, we propose that it is necessary to set the appropriate dosages of the two vectors in the HSV-tk plus MCP-1 system.

Recently, we found that the HSV-tk/GCV system, together with delivery of MCP-1, eradicated HCC and exerted prolonged antitumor effects by activating macrophages and NK cells.[20] In this study, the antitumor immunity increased in mice treated with Ad-tk plus Ad-MCP1 (1:0.1). Several investigators have reported that dying HSV-tk-modified cells released soluble factors, including cytokines.[27,28] These factors could in turn affect the tumor microenvironment, leading to necrosis and inflammation, infiltration of immune cells, up-regulation of costimulatory molecules, and generation of an antitumorigenic immune responses.[28,29] In this immunotherapeutically favorable setting, the optimal dose of MCP-1 with HSV-tk inside the same cell may stimulate tumor-specific immune-mediated cell killing. Consequently, the delivery of an optimal amount of rAd expressing MCP-1 enhanced the antitumor effects of the HSV-tk/GCV system in a model of HCC, and the effects were related to the balance of Th1 and Th2–type cytokines.

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Original Article

Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis

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Aim: Regulatory T cells (Tregs) maintain immunological tolerance and suppress autoreactive immune responses. We evaluated the intrahepatic status of Tregs in patients with autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), or chronic hepatitis B (CH-B).

Methods: We analyzed 85 patients (20 AIH, 22 PBC, 27 CH-C, and 16 CH-B) and 14 controls. Using liver tissue samples obtained by needle biopsy or from marginal parts of resected metastatic liver tumors in the controls, immunohistochemical analyses of forkhead box P3$^+$, which is a specific marker for Tregs, CD4$^+$, and CD8$^+$ cells were performed.

Results: Intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls. There were significantly fewer intrahepatic Tregs in the AIH patients than in the PBC patients ($P = 0.037$). Patients with a low frequency of intrahepatic Tregs were detected significantly more in the AIH and CH-B groups than in the PBC and CH-C groups ($P < 0.05$). In addition, the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced. We found significantly less infiltration of CD4$^+$ T cells in AIH than in other diseases ($P < 0.05$). Liver-infiltrating CD8$^+$ T cells were detected more frequently in the CH-B group than in other groups ($P < 0.003$).

Conclusion: Intrahepatic Tregs were increased in both patients with autoimmune liver diseases and those with viral hepatitis. In autoimmune liver diseases, intrahepatic Tregs were fewer in the AIH patients than in the PBC patients.

Key words: autoimmune hepatitis, chronic hepatitis, forkhead box P3, primary biliary cirrhosis, regulatory T cells

INTRODUCTION

T-CELL RESPONSES are implicated in host immune defense against microbes as well as immunopathogenesis of certain diseases, such as viral hepatitis. An appropriate T-cell response leads to the eradication of microbes, while a weak response may result in persistent infection. If the T-cell activation is too potent, however, severe inflammation or autoimmune disease may develop. The detailed mechanisms that lead to the breakdown of self-tolerance and the subsequent development of autoimmune disease are still unknown; however, the mechanisms are likely to involve the failure of homeostatic processes that keep the response against self-antigens under control.[1]

T-cell populations regulate and control the balance of immune responses. The CD4$^+$ and CD25$^+$ regulatory T cells (Tregs) are crucial for maintaining immunologic self-tolerance and negative control of various immune responses. The majority of Tregs are produced by the thymus as a functionally distinct T-cell subpopulation and are responsible for maintaining peripheral tolerance. Genetic abnormalities in the development and function of this Treg population can cause autoimmune disease, immunopathology, and allergy in humans.[2] In addition, there are different T-cell subpopulations with regulatory functions, such as natural killer T cells, T helper 3, T regulatory 1, CD8$^+$ and CD28$^-$, and $\gamma\delta$ T cells. These types of T cells may also prevent the activation of autoreactive T cells and be involved in the failure of homeostasis.[1]

Although several cell-surface molecules, such as CD25, glucocorticoid-induced tumor necrosis factor

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receptor family-related gene/protein, and cytotoxic T lymphocyte-associated molecule-4, have been reported as Treg markers, these molecules are also expressed on activated T cells derived from CD4$^+$ and CD25$^-$ naïve T cells.[3] Transcription factor forkhead box P3 (FOXP3) is expressed in CD4$^+$ and CD25$^+$ Tregs as a master control molecule for their development and function in mice and humans, thus, FOXP3 is thought to be a specific marker of Tregs.

Autoimmune mechanisms are involved in autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC). AIH is an inflammatory liver disease characterized by high levels of transaminases, circulating autoantibodies, hyper-γ-globulinemia, histological evidence of interface hepatitis, and response to immunosuppressive treatment.[4,5] PBC is an enigmatic liver disease characterized by the chronic non-suppurative destruction of small intrahepatic bile ducts, portal inflammation, and the presence of antimitochondrial antibodies (AMA).[6,7] The presence of AMA and autoreactive T and B cells, in conjunction with the co-occurrence of other autoimmune diseases, characterizes PBC as a typical autoimmune disease.[8] Although the etiology of PBC remains obscure, recent data suggest that autoreactive T-cell responses play a major role in its pathophysiology.[9–12]

Hepatitis C virus (HCV) infection is often asymptomatic, and approximately 80% of infected patients progress to chronic hepatitis.[13] After HCV infection, interaction between the innate and adaptive immune responses plays a pivotal role in perpetuation or clearance of HCV infection. T helper 1–type (Th1) cytokines, such as interferon (IFN)-γ and interleukin (IL)-2, are involved in cell-mediated immunity and play a crucial role in protection against intracellular pathogens.[14] A weak cellular immune response is thought to be one of the mechanisms of HCV persistence.

In hepatitis B virus (HBV) infection, a multispecific CD4$^+$ and CD8$^+$ T cell with a Th1 cytokine profile is also important for control of the infection.[15] These multispecific T-cell responses are maintained for decades after clinical recovery. However, these responses are lacking in patients with chronic HBV infection, and the mechanism of T-cell hyporesponsiveness or tolerance is still unknown.

The frequency of Tregs in the peripheral blood was decreased in patients with AIH and PBC and increased in patients with chronic hepatitis C (CH-C) and chronic hepatitis B (CH-B) compared with the healthy controls.[16] However, there are few reports investigating the intrahepatic status of Tregs. In the present study, we analyzed and compared the intrahepatic status of Tregs in patients with AIH, PBC, CH-C, and CH-B because liver-infiltrating immune cells should reflect the status of disease and pathogenesis more directly than peripheral cells.

METHODS

Patients and liver tissue

NEEDLE BIOPSIES WERE performed to obtain liver tissue from 85 patients, consisting of 20 AIH patients, 22 PBC patients, 27 CH-C patients, and 16 CH-B patients. All patients had a persistently increased level of serum alanine aminotransferase (ALT; >30 IU/L). The diagnosis of each case was based on reliable clinical and laboratory data and was independently confirmed histologically by two pathologists who specialize in liver diseases. All AIH patients were antinuclear antibody positive or antismooth muscle antibody positive, and all had histological features of interface hepatitis. Patients with morbid changes in bile duct were excluded individually by retrograde radiological cholangiography or magnetic resonance cholangiopancreatography. Patients with overlap syndrome were also excluded from this study. All PBC patients were AMA positive and fulfilled the diagnostic criteria of PBC based on internationally accepted standards. Livers from PBC patients were staged histologically by Scheuer's classification. Seventeen and five patients were of stage 1 and of stages 2/3/4, respectively. We included 14 patients with metastatic liver tumors as the controls. The control patients were not infected with HBV (negative for hepatitis B surface antigen) or HCV (negative for anti-HCV antibody), and they had no history of autoimmune diseases and were negative for autoimmune antibodies. Liver tissue from control patients was obtained from a marginal part of the resected liver in which the histological examination was normal. Table 1 shows the patients' characteristics. All patients gave written informed consent according to a protocol approved by the Ethical Committee of Showa University.

Immunohistochemical staining

Liver needle biopsies and resected tissues were obtained from the 99 patients, as described earlier. All tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 3 µm–thick serial sections were cut from each paraffin block. Each specimen contained at least three portal tracts encompassing interlobular bile ducts, and a total of 297 portal tracts were counted. Antigen retrieval for CD4 and FOXP3 staining

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Table 1 Characteristics of the patients and controls analyzed in this study

Group (number)	AIH (20)	PBC (22)	CH-C (27)	CH-B (16)	Control (14)
Female (%)	95.0[*,**,†,††]	90.9[‡,‡‡,¶]	40.7	18.8	28.6
Age (years)	55.8 ± 13.8[†]	55.5 ± 11.2[‡‡]	49.8 ± 11.9[§,§§]	38.1 ± 11.6[‖]	59.1 ± 14.2
ALT (IU/L)	393 ± 462[*,**,†,††]	113 ± 139[‡‡,¶]	85 ± 57[§,§§]	295 ± 351[‖]	17 ± 9
AST (IU/L)	306 ± 393[*,**,†,††]	88 ± 78[¶]	60 ± 40[§,§§]	148 ± 141[‖]	22 ± 9
ALP (IU/L)	495 ± 300[*,**,†]	842 ± 547[‡,‡‡,¶]	268 ± 85	320 ± 96	316 ± 171
IgG (mg/dL)	2639 ± 1163[*,**]	1837 ± 584	1794 ± 290	n.d.	n.d.
IgM (mg/dL)	334 ± 355[**]	466 ± 231[‡]	129 ± 67	n.d.	n.d.

Significance was assessed with Fisher's exact probability test. $P < 0.05$ (*AIH versus PBC, **AIH versus CH-C, †AIH versus CH-B, ††AIH versus Control, ‡PBC versus CH-C, ‡‡PBC versus CH-B, ¶PBC versus Control, §CH-C versus CH-B, §§CH-C versus Control, ‖CH-B versus Control). Values are mean ± standard deviation. AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; IgG, immunoglobulin G; IgM, immunoglobulin M; n.a., not determined; PBC, primary biliary cirrhosis.

was achieved by pressure cooking for 5 min in citrate buffer (pH 7.0), while antigen retrieval for CD8 staining was achieved by microwaving for 15 min in citrate buffer (pH 7.0). For CD4 or CD8 immunohistochemical staining, anti-CD4 monoclonal antibody (mAb; Nichirei Biosciences, Tokyo, Japan) or anti-CD8 mAb (Dako Cytomation, Tokyo, Japan) and biotinylated goat antimouse immunoglobulin G (IgG; Dako ChemMate Envision kit/HRP[DAB], Dako, Japan) were used. FOXP3 expression was analyzed by immunostaining with a goat antihuman FOXP3 polyclonal antibody (ab22510; Abcam, Cambridge, UK) and biotinylated rabbit antigoat IgG (Dako ChemMate Envision kit/HRP[DAB]). The slides were stained with hematoxylin following immunohistochemical staining.

Evaluation of frequency of FOXP3-, CD4-, and CD8-positive cells

To evaluate and compare the distribution and frequency of cells positive for FOXP3, CD4, and CD8, three small- to medium-sized portal tract areas were selected for investigation with an optical microscope. The same visual fields were chosen and examined using serial sections. The numbers of FOXP3-, CD4-, or CD8-positive cells contained within the three portal tract areas from each specimen were counted at a magnification of ×400 by two independent observers in a blinded fashion. To correct for differences in the sizes of the portal tracts, the proportion of $FOXP3^+$ Tregs was determined as follows: %FOXP3 = (counts of $FOXP3^+$ Tregs/counts of total mononuclear cells) × 100, which is a total mononuclear cell-corrected value for $FOXP3^+$. $CD4^+$ and $CD8^+$ T cells in total mononuclear cells were also calculated.

Statistical analyses

Significance was assessed with the Mann–Whitney *U*-test or Fisher's exact probability test. Differences between groups were considered statistically significant when the *P*-value was less than 0.05.

RESULTS

Intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls

TO COMPARE THE frequencies of intrahepatic $FOXP3^+$ Tregs between the liver diseases, we determined the percentage of FOXP3, as described in Methods. As shown in Figure 1, the frequency of $FOXP3^+$ T cells in patients with AIH, PBC, CH-C, or CH-B was significantly much higher than that in the control patients. Interestingly, there were significantly fewer $FOXP3^+$ T cells in the liver tissues of AIH patients than in those of PBC patients ($P = 0.037$). The frequency of intrahepatic $FOXP3^+$ Tregs in the AIH patients was not different from that in the patients with CH-C or CH-B.

Patients with a low frequency of intrahepatic Tregs were detected significantly more in the AIH and CH-B groups than in the PBC and CH-C groups

Since the patients with numerous intrahepatic $FOXP3^+$ Tregs were observed in the PBC and CH-C groups, we separated the patients into two groups according to the frequency of intrahepatic Tregs. The patients were divided into those with $FOXP3^+$ cells of less than 9% and those with $FOXP3^+$ cells of 9% or more: this level

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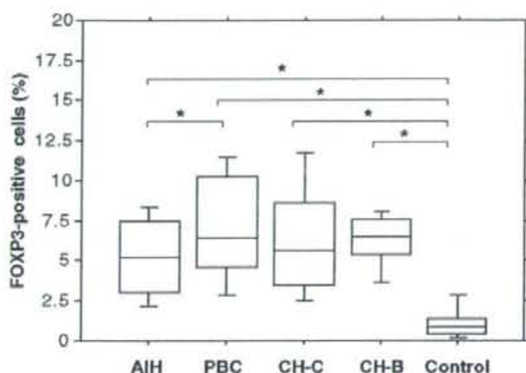

Figure 1 Intrahepatic forkhead box P3$^+$ (FOXP3$^+$) T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic FOXP3$^+$ cell status, intrahepatic FOXP3$^+$ cells in patients with AIH, PBC, CH-C, and CH-B were stained. For the enumeration of positive mononuclear cells, mononuclear cells were counted in three high-powered fields (×400) by two independent observers in a blinded fashion. For each sample, the mean percentage of positive cells was chosen. Results are expressed as the median and range of all tested patents in each group. $P < 0.05$.

was decided arbitrarily as follows: (mean percentage of FOXP3 of controls + 3 standard deviation) × 2. When the number of patients with a high frequency of intrahepatic Tregs (%FOXP3 ≥9%) and that with a low frequency (%FOXP3 <9%) were compared for each liver disease, patients who had a low frequency of intrahepatic Tregs were detected significantly more in the AIH group than in PBC and CH-C groups as shown in Table 2. In addition, more patients with low frequency Treg infiltration were found in the CH-B group than in the PBC and CH-C groups. Thus, PBC is characterized by higher frequency of FOXP3$^+$ cells compared to AIH, whereas the number and profiles of liver-infiltrating T cells are comparable. In viral hepatitis, a higher frequency of FOXP3$^+$ cells is observed in CH-C, while higher frequency of CD4$^+$ or CD8$^+$ cells is characteristic for HBV-infected liver.

Table 3 Comparison of the intrahepatic Tregs frequency with histological stages in PBC patients

	<9%	≥9%	Total
Early stage	8	9	17
Advanced stage	5	0	5
Total	13	9	22

$P = 0.034$. PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4). Number of patients with a high frequency of intrahepatic regulatory T cells (Tregs; %FOXP3 ≥9%) and that with a low frequency (%FOXP3 <9%) were compared for each stage.
PBC, primary biliary cirrhosis.

When the PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4), the frequency of Tregs was higher than 9% in nine of 17 (53%) PBC patients with early histological stage, while that of Tregs was below 9% in all patients with advanced stage ($P = 0.034$), as shown in Table 3. Furthermore, as shown in Figure 2, more FOXP3$^+$ T-cell infiltration was seen in the early stage than in the advanced stage (8.03 ± 3.50 *vs* 4.47 ± 1.40, $P = 0.041$). Therefore, it was thought that the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced.

Frequency of intrahepatic CD4$^+$ T cells was lower in AIH patients, while the frequency of intrahepatic CD8$^+$ T cells was higher in CH-B patients

We evaluated the intrahepatic frequencies of CD4$^+$ cells as well as CD8$^+$ cells to investigate whether these

Table 2 Comparison of the number of patients with high frequency of intrahepatic Tregs with those with low frequency

	<9%	≥9%	Versus PBC*	Versus CH-C**
AIH ($n = 20$)	20	0	$P = 0.001$	$P = 0.014$
PBC ($n = 22$)	13	9	–	$P = 0.266$
CH-C ($n = 27$)	20	7	$P = 0.266$	–
CH-B ($n = 16$)	16	0	$P = 0.003$	$P = 0.026$
Control ($n = 14$)	14	0	$P = 0.006$	$P = 0.036$

Significance was assessed with Fisher's exact probability test. P-values are shown as VS PBC group(*) and VS CH-C(**) group.
AIH, autoimmune hepatitis; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; PBC, primary biliary cirrhosis; Tregs, regulatory T cells

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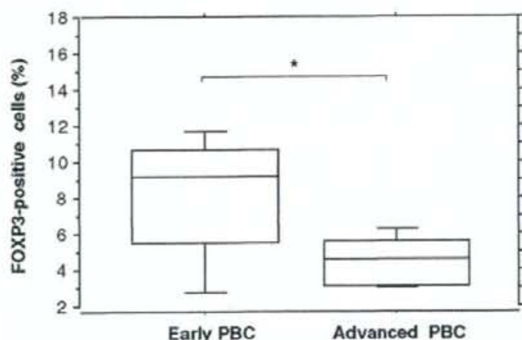

Figure 2 Frequency of regulatory cells (Tregs) in the liver of primary biliary cirrhosis (PBC) patients in terms of pathological stage of the disease advances. Intrahepatic forkhead box P3+ (FOXP3+) T cells in the PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4). Results are shown as the mean percentage of Tregs frequency ± standard deviation in each stage. $P < 0.05$.

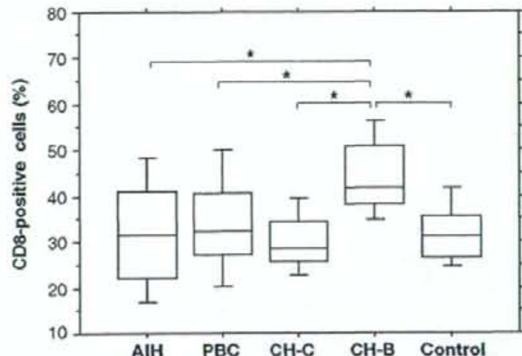

Figure 4 Intrahepatic CD8+ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD8+ cell frequency, intrahepatic CD8+ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD8+ cells were counted with the same procedure used for forkhead box P3+ cells. Results are expressed as the median and range of all tested patents in each group. $P < 0.05$.

immune cells were involved in the immunopathogenesis of each liver disease. As shown in Figure 3, the frequency of CD4+ T cells infiltrating the liver tissue was significantly higher in CH-B patients than in the controls. We found significantly less infiltrating CD4+ T

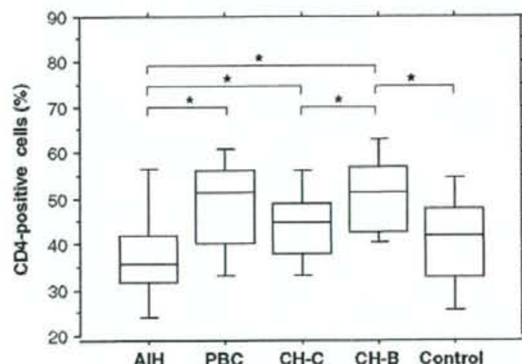

Figure 3 Intrahepatic CD4+ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD4+ cell frequency, intrahepatic CD4+ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD4+ cells were counted with the same procedure used for forkhead box P3+ cells. Results are expressed as the median and range of all tested patients in each group. $P < 0.05$.

cells in the AIH patients than in the PBC patients ($P = 0.007$), CH-C patients ($P = 0.045$), and CH-B patients ($P < 0.001$). As shown in Figure 4, the frequency of CD8+ T cells was significantly higher in the CH-B patients than in the controls. There were also significantly higher CD8+ T cells in the liver tissues of the CH-B patients than in those of the AIH patients ($P = 0.003$), PBC patients ($P = 0.002$), and CH-C patients ($P < 0.001$).

Furthermore, the CD4+/CD8+ ratio was lower in the CH-B patients than in the PBC patients (1.18 ± 0.26 *vs* 1.56 ± 0.66, $P = 0.037$) and CH-C patients (1.18 ± 0.26 *vs* 1.49 ± 0.39, $P = 0.007$). There was no difference in the total infiltration of mononuclear cells between patients with AIH, PBC, CH-C, and CH-B. Intrahepatic CD4+ T cells and CD8+ T cells in the control patients were significantly less than in the CH-B patients ($P = 0.013$ and $P < 0.001$, respectively), although we did not detect any differences between the control group and the other liver disease groups. There was no relationship between the biochemical data or histological activities and infiltration of the immune cells.

Since intrahepatic immune cells may directly affect inflammation in the liver, we compared the biochemical data, such as the serum ALT level, and histological activities with the intrahepatic frequencies of FOXP3+, CD4+, and CD8+ cells. There was no relationship between the ALT, alkaline phosphatase, IgG, immuno-

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globulin M level, or histological activities and the frequency of infiltrating immune cells other than described above (data not shown).

DISCUSSION

TREGS ARE THOUGHT to play roles in immune regulation, such as the suppression of severe inflammation and autoimmune diseases. The removal or reduction of Tregs can also enhance immune responses against infectious microbes, thus, Tregs affect the elimination of infectious microbes.[17–24] A higher proportion of $CD4^+$ and $CD25^+$ T cells in peripheral blood was found in patients with chronic HCV infection as compared to recovered patients and normal controls.[25] Tregs secrete transforming growth factor-β_1 and IL-10, and these cytokines may attenuate the function of macrophages. IL-10 also inhibits HCV-specific immunity when administered exogenously in patients with chronic HCV infection.[26] Thus, Tregs may disturb the eradication of HCV and lead to chronic infection. Chronic HBV patients harbor an increased frequency of Tregs in peripheral blood as compared to control patients, and Tregs have an immunosuppressive effect on HBV-specific T helper cells.[27] This may be one of the mechanisms that leads to chronic infection.

Several recent studies have focused on Tregs in patients with autoimmune liver diseases, such as AIH and PBC. Since Tregs prevent the proliferation and effector function of autoreactive T cells[16] and downregulate the production of IFN-γ by $CD8^+$ T cells in a murine model and in humans,[28,29] Tregs may be implicated in the pathogenesis of AIH and PBC. In fact, the relative frequencies of Tregs are decreased in peripheral blood samples of patients with PBC,[30] and Tregs are few in patients with AIH.[16] However, there are only a few reports regarding the status of intrahepatic Tregs.

Tregs maintain the ability to suppress IFN-γ production by $CD4^+$ and $CD25^-$ T cells in AIH, and circulating Tregs are significantly less in AIH patients than in controls.[16] However, few details regarding the roles of Tregs in the pathogenesis of AIH have been revealed.

In the present study, we demonstrated that intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls. Indeed there are significantly fewer intrahepatic Tregs in AIH patients and CH-B patients than in PBC patients and CH-C patients, but as a whole, there is more infiltration of $FOXP3^+$ Tregs than in the controls, and there is not a great difference. In addition, we found significantly fewer infiltrating $CD4^+$ T cells in AIH patients than in the patients with other diseases, whereas $CD8^+$ T cells infiltrating liver tissue were detected with a significantly greater frequency in CH-B patients than in the other patients.

Although both AIH and PBC are representative autoimmune liver diseases, we identified differences in immune cell infiltration between these two autoimmune diseases in the present study. The results indicate that different mechanisms are involved in the pathogenesis of AIH and PBC. However, there are significantly more ratios of Tregs than control, and it seems that only a ratio of Tregs does not relate to the pathogenesis of these diseases.

We found that the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced. A previous report demonstrated that there were few liver-infiltrating Tregs in PBC patients,[30] although it has not been confirmed by other researches. Sasaki *et al.* recently reported findings similar to ours. They found that the extent of $FOXP3^+$ Tregs in inflamed portal tracts with chronic non-suppurative destructive cholangitis in early stage (Scheuer's classification 1 and 2) of PBC was higher than that in late stage (Scheuer's classification 3, 4) of PBC.[31]

It is not clear whether this decrease of Tregs is a cause or a result of disease progression. Although we cannot explain the reason for these differences in Tregs' infiltration, the race of the study patients may be one of the factors. Functional investigations of intrahepatic Tregs in these autoimmune liver diseases may clarify this issue.

Since the frequency of intrahepatic Tregs in CH-C groups is diverse widely, we could not detect a significant difference in Tregs' accumulation between the CH-C and AIH groups. However, several CH-C patients had a large number of intrahepatic Tregs. When we divided the patients in each group into those with $FOXP3^+$ cells of less than 9% and those with $FOXP3^+$ cells of 9% or more, a significant difference was confirmed. In addition, patients who had a high frequency of intrahepatic Tregs were detected significantly more often in the CH-C group than in the CH-B group. In HCV infection, it has been suggested that HCV itself, especially in the NS3 region, induces Tregs in patients with HCV infection as well as in healthy donors,[32] and these Tregs are involved in the development of viral persistence, which occurs usually in acute HCV infection and rarely in acute HBV infection in adults. Thus, in chronic hepatitis, the pathogenesis of HCV should be different from that of HBV.

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There were only a few Tregs in the pathologically normal tissue that surrounded metastatic liver tumors. The same phenomenon has been described in other reports.[30,33] The decreased frequency of Tregs was not likely to be the effect of metastatic tumors, because it has been reported that malignant tumors often induce Tregs.[34–39] In normal liver tissue, Treg infiltration may be suppressed because it is necessary to induce immunity against many pathogens flowing into the liver, rather than prevent inflammation or induction of autoimmunity.

Intrahepatic Tregs may be involved with immunopathogenesis and play a crucial direct role in the development of each liver disease. However, since immune systems in liver diseases are complicated, further investigations are needed to clarify the detailed relationship between Tregs and immunopathogenesis.

ACKNOWLEDGMENT

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MINI-SERIES REVIEW: BASIC SCIENCE OF GASTROENTEROLOGY AND HEPATOLOGY

Immune responses in hepatitis C virus infection and mechanisms of hepatitis C virus persistence

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Key words
cytotoxic T lymphocyte, dendritic cell, helper T cell, natural killer cell, regulatory T cell.

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Abstract

Immune responses against hepatitis C virus (HCV) play a crucial role in the pathogenesis of chronic hepatitis C. HCV infection often persists and leads to chronic hepatitis and eventually cirrhosis. Accumulated data suggest that HCV proteins suppress host immune responses through the suppression of functions of immune cells, such as cytotoxic T lymphocytes, natural killer cells, and dendritic cells. They also suppress the type 1 interferon signaling system. The resulting insufficient immune responses against HCV lead to the sustained infection. The appropriate control of immune responses would contribute to the eradication of HCV and the improvement of hepatitis, but there are still many issues to be clarified. This review describes the scientific evidence to support these emerging concepts, and will touch on the implications for improving antiviral therapy.

Introduction

Hepatitis C virus (HCV) infection often persists and causes chronic hepatitis that may progresses to cirrhosis, followed by the development of hepatocellular carcinoma. HCV infection is a worldwide health problem. HCV was first identified by Chiron's group in 1989,[1] following sustained attempts by many researchers to find by various methods to isolate the causative agent of the so-called non-A, non-B hepatitis. In 1989, one of our authors (MI) established cytotoxic T-lymphocyte (CTL) clones, which recognize hepatocytes from patients with non-A, non-B hepatitis, but not those of donors without non-A, non-B hepatitis.[2] Unfortunately, we were unable to clarify the target protein recognized by the clones. The discovery of HCV contributed much to the diagnosis of hepatitis C, and proved that HCV was the causative agent of most of chronic non-A, non-B hepatitis. It was also a breakthrough in investigations of the immune responses to HCV infection and the immunopathogenesis of chronic hepatitis C. Hepatitis viruses themselves are not cytopathic to hepatocytes directly. Rather, hepatitis is caused by host immune cells, such as natural killer (NK) cells and CTL, which attack and destroy virus-infected hepatocytes to eradicate viral infections. It has been reported that CTL, which recognize peptides derived from hepatitis viruses, exist in the liver and the peripheral blood.[3] CTL specific for particular hepatitis viruses are thought to be one of the major host defense mechanisms, and have been implicated in viral clearance and the immunopathogenesis of viral hepatitis.[4]

In this review, cellular immune responses against HCV, the immunopathogenesis of HCV infection, and the escape mechanisms of HCV from immunosurveillance are summarized.

Host immune responses in HCV infection and immunopathogenesis of hepatitis C

Non-specific immune response

As in other viral infections, non-specific immune responses are induced in the host infected by HCV (Fig. 1). In the early stages of HCV infection, type I interferons (IFN; α/β) are produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DC). Type I IFN induce the expression of 2′–5′ oligoadenylate synthetase which inhibits HCV replication. Type I IFN enhance the expression of human leukocyte antigen (HLA) class I on the surface of antigen-presenting cells, such as DC, and reinforce cellular immune responses. The latter include the activation of NK cells and CTL. NK cells activated by type I IFN injure HCV-infected hepatocytes, and this process initiates hepatitis. In turn, the destruction of hepatocytes stimulates myeloid DC. Subsequently, these DC promote the secretion of a high amount of IFN-γ through the activation of NK cells and NKT cells; the latter having characteristics of both NK cells and T cells, and exist mainly in the liver. IFN-γ then activates hepatic macrophages to enhance local inflammation.

HCV-specific immune response

When the host is unable to eradicate HCV despite the induction of non-specific immune responses, HCV-specific immune responses are induced to eliminate the residual HCV.

Figure 2 shows the process of inducing HCV-specific immunes. Type 1 helper T (Th1) cells play a crucial role in the induction and

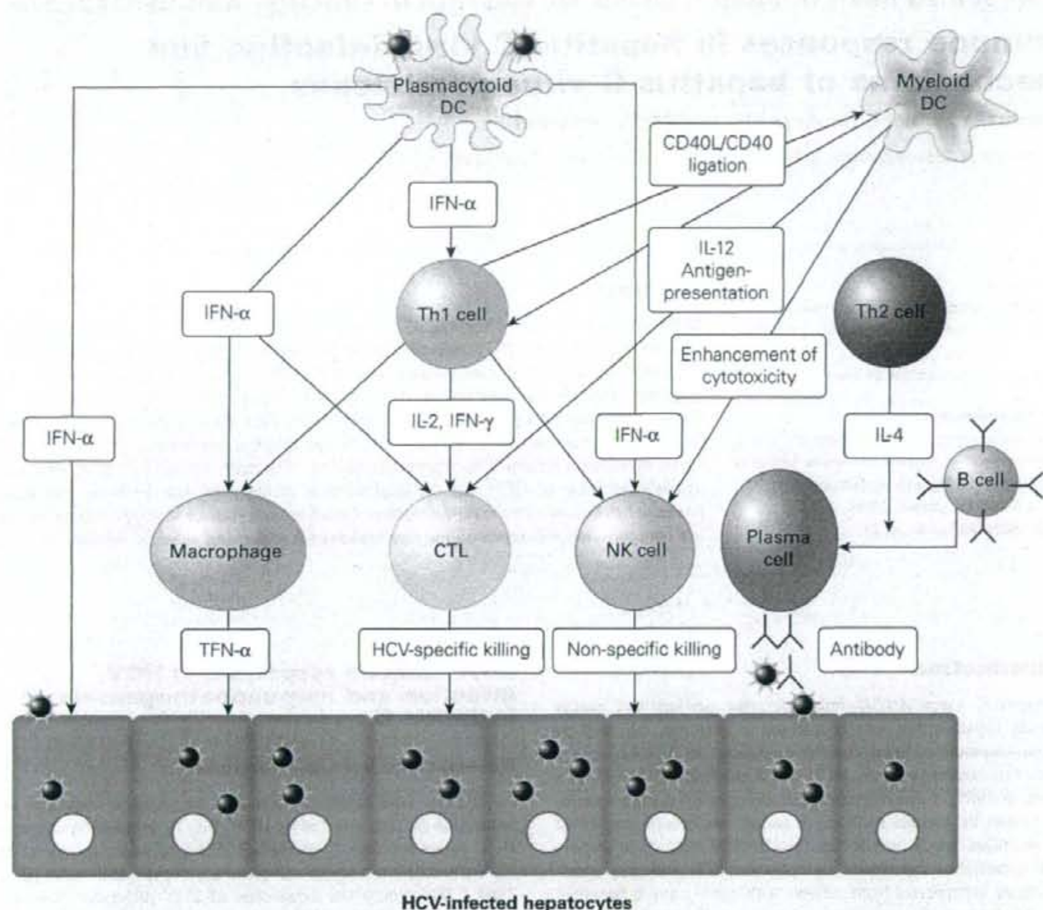

Figure 1 Immune responses in hepatitis C virus (HCV) infection. In the early stage of HCV infection, interferon (IFN)-α is produced by plasmacytoid dendritic cells (DC). IFN-α activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTL). NK cells activated by type I IFN injure the HCV-infected hepatocytes in a non-specific manner, whereas CTL do so in an antigen-specific manner. Destruction of hepatocytes stimulates myeloid DC. These DC secrete a high amount of interleukin (IL)-12, which promotes the activation of NK cells and type 1 helper T (Th1) cells. Activated Th1 cells in turn promote DC maturation by CD40/CD40 ligand (CD40L) interaction. Plasma cells secrete immunoglobulins to neutralize circulating HCV. Th2 cell, type 2 helper T cell.

activation of virus-specific cellular immune responses, such as CTL. After taking up HCV antigens in the liver, myeloid DC move to a draining lymph node. As they mature at the lymph node, the expression of HLA and costimulatory molecules is upregulated on their surface. These matured DC activate naïve helper T (Th) cells efficiently through stimulation with HLA class II and costimulatory molecules. In turn, the stimulated Th cells further activate DC by the expression of the CD40 ligand, as well as the secretion of cytokines, such as tumor necrosis factor (TNF)-α. Interleukin (IL)-12, produced mainly by myeloid DC, differentiates these stimulated Th cells towards Th1 cells. Activated Th1 cells secrete IL-2 and IFN-γ, which induce the activation and proliferation of CTL and NK cells. As a continuation of this process, naïve CTL recognize HCV antigens presented on the DC, and the stimulated HCV-specific CTL leave the lymph nodes for the liver. They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and eradicate HCV by killing the infected hepatocytes.

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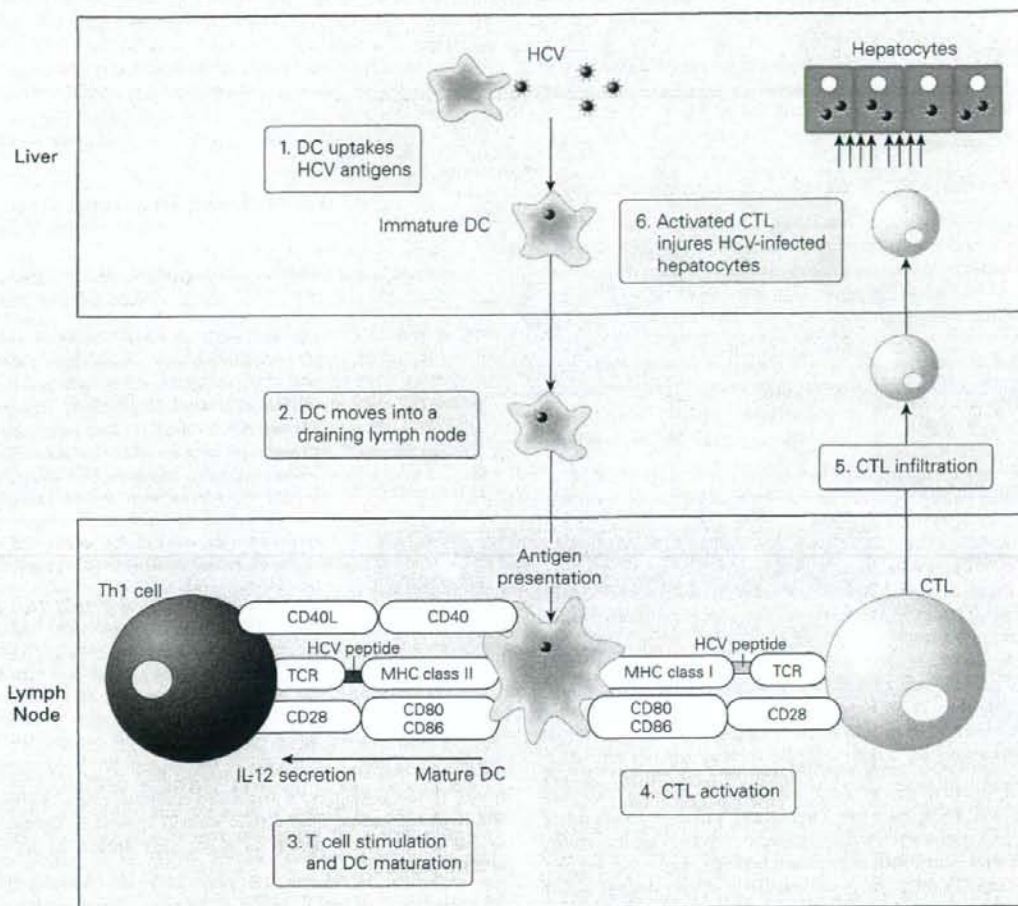

Figure 2 Induction of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL). After taking up HCV antigens in the liver, myeloid dendritic cells (DC) move to a draining lymph node. Matured DC activate naïve helper T (Th) cells efficiently through stimulation with HLA class II and costimulatory molecules (CD80 and CD86). Stimulated Th cells in turn further activate DC by the expression of the CD40 ligand (CD40L) as well as secretion of tumor necrosis factor-α. Interleukin (IL)-12 produced by myeloid DC differentiates these stimulated Th cells towards Th1 cells. IL-2 and interferon-γ secreted by activated Th1 cells induce the activation and proliferation of CTL and natural killer NK cells. As a continuation of this process, naïve CTL recognize HCV antigens presented on the DC, and the stimulated HCV-specific CTL leave the lymph nodes for the liver. They recognize HCV antigens together with human leukocyte antigen (HLA) class I on the surface of HCV-infected hepatocytes, and eradicate HCV by killing the infected hepatocytes. MHC, major histocompatibility complex; TCR, T-cell receptor.

It has been reported that virus-specific CTL play an important role in viral eradication because CTL not only kill HCV-infected cells, but also inhibit viral replication. Since we first identified an HLA B44-restricted CTL epitope,[3,5] we have identified more than 20 CTL epitopes along the HCV polyprotein using synthetic peptides and enzyme-linked immunospot assay.[6,7] When peripheral blood lymphocytes of patients with HCV infection were stimulated with synthetic peptides corresponding to the CTL epitopes, we could frequently detect CTL responses in patients who had been infected with HCV within the past 3 years, but hardly ever in patients infected with HCV more than 10 years ago. It has been reported that patients who had spontaneously eradicated HCV infection up to 35 years earlier demonstrated persistent CD4+ and CD8+ T-cell responses specific to HCV peptides.[8]

At the time of HCV infection, if appropriate cellular immune responses are induced, viruses can be completely eradicated. In

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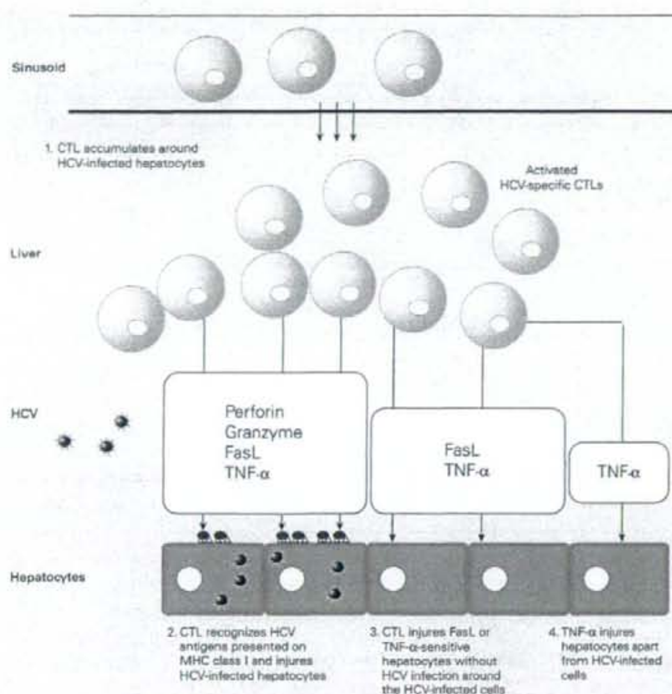

Figure 3 Killing mechanisms of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) on hepatocytes. Activated HCV-specific CTL stimulated by matured dendritic cells (DC) infiltrate the liver tissue through a sinusoid. They recognize HCV antigen peptides and release perforin and granzyme. Activated CTL are also involved in killing hepatocytes through the expression of Fas ligands and the secretion of tumor necrosis-α (TNF-α). Hepatocytes in patients with severe hepatitis show increased susceptibility as a result of enhanced expression of Fas and/or TNF-α receptors. Activated CTL injure compromised hepatocytes, which have acquired sensitivity to the Fas ligand (FasL) and TNF-α, even if they are not infected with HCV. TNF-α released from activated CTL can injure compromised non-infected cells without cell-to-cell interaction.

HCV infection, however, immune responses are often not potent enough to eliminate the virus, and the weak immune responses result in relatively mild liver damage and lead to persistent infection. Conversely, when induced immune responses are too strong, severe liver injury, such as fulminant hepatitis, may occur, but this is rare in HCV infection. A moderate level of liver damage would occur through a balance between viral load and host immune responses.

It has also been reported that activated HCV-specific CTL accumulate in the liver of patients with chronic hepatitis C, and that they are involved in the inhibition of viral replication and liver damage. Given that CTL responses were frequently detectable in the peripheral blood of patients with a low titer of serum HCV-RNA, it was suggested that HCV-specific CTL may inhibit the replication of HCV, and that infection with high-titer HCV may suppress the CTL response.[9] Chronic hepatitis C patients with an HCV-specific CTL response had significantly high levels of serum alanine aminotransaminase, but there was no relationship between the CTL response, the clinical course, or the pathological severity.[10]

An extremely vigorous CTL response specific to a single epitope (HCV NS3 1073-1081), which cross-reacted with an influenza neuraminidase sequence, could be detected in patients with severe hepatitis, but not with mild hepatitis.[11] From this result, it was suggested that CTL cross-reactivity affects the severity of HCV-associated liver injury. A mutation of this CTL epitope at residues 1073–1081 of the NS3 protease may allow for escape from CTL recognition, but it would also diminish protease activity and RNA replication. Thus certain variants capable of immunological escape rarely appear as major viral species in HCV-infected patients.[12]

Killing mechanisms of HCV-infected hepatocytes by specific CTL

Activated HCV-specific CTL stimulated by matured DC infiltrate the liver tissue through a sinusoid. They recognize HCV antigen peptides, which consist of 8–11 amino acids, presented by HLA class I molecules through a T-cell receptor, and release perforin, which is inserted into the target cell's plasma membrane. This forms a pore and leads to the uptake of granzyme, which induces apoptosis of HCV-infected cells (Fig. 3). Activated CTL are also involved in killing hepatocytes through the expression of the Fas ligand and secretion of TNF-α. Perforin induces cell death in almost all cells, whereas the Fas ligand and TNF-α do so only in cells displaying their cognate receptors (Fas, TNF-R1). Normal hepatocytes show resistance against the Fas ligand and TNF-α while hepatocytes in patients with severe hepatitis show increased susceptibility as a result of the enhanced expression of Fas and/or TNF-α receptors. Although the cytotoxic activity of the Fas ligand and TNF-α is less potent than that of perforin, activated CTL

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