Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CP§	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.

†Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).

‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).

§Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2). In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than "other substitutions" (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

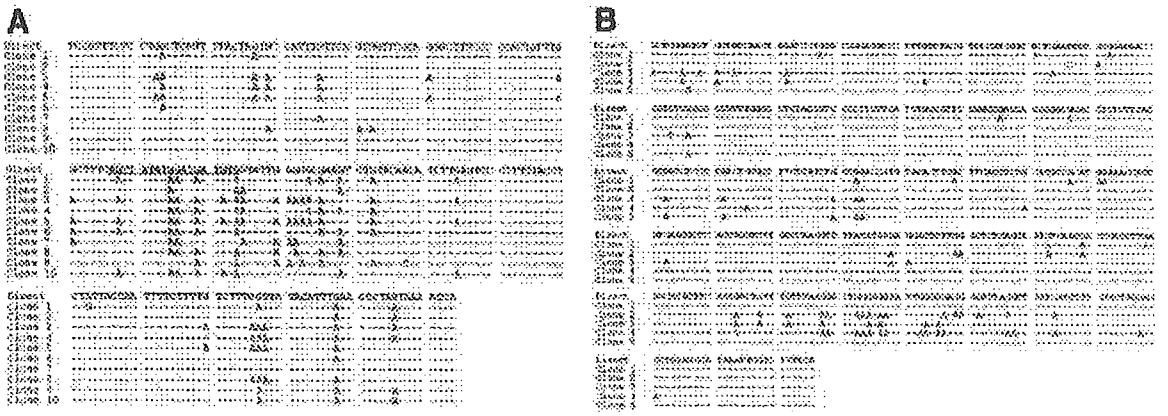

Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (**top line**). The target sequence of PNA annealing is **underlined**. (B) DNA sequence alignment in the x region of the HBV.

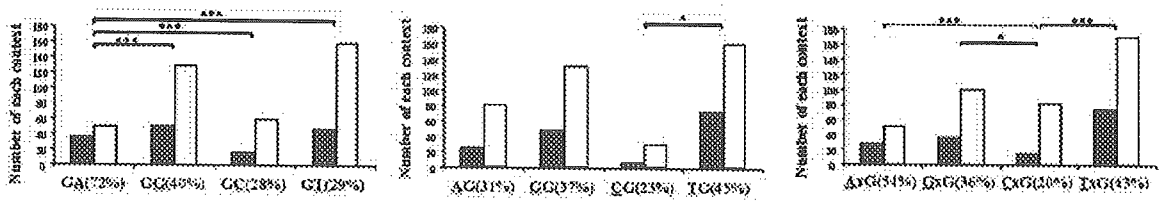

Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. **Open bars**: number of occurrences of each context in the sequence analyzed. **Gray bars**: number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues.*$P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions*		No. of Clones†	eAg	HBs Ag	HBV DNA
	G to A	Other				
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

Expression of Deaminases in HepG2 Cell Lines. We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.[23] The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.[23] The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,[21] we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

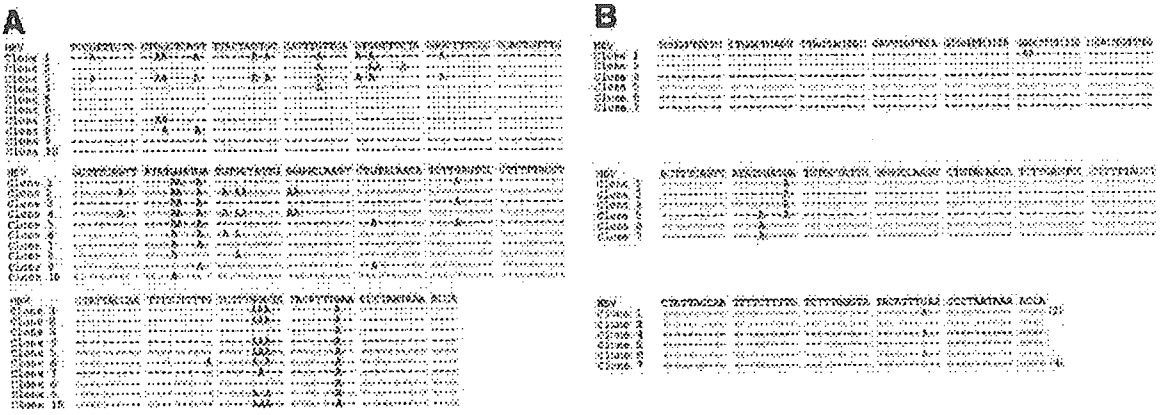

Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (**top line**). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,[19] in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.[24-28] However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOVEC3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.[20,29] suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.[30] reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,[30] suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

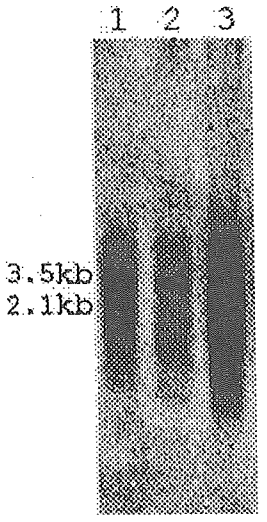

Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

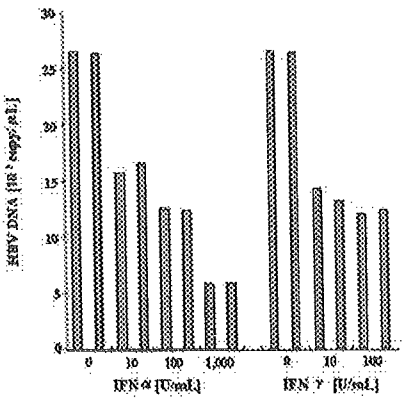

Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.

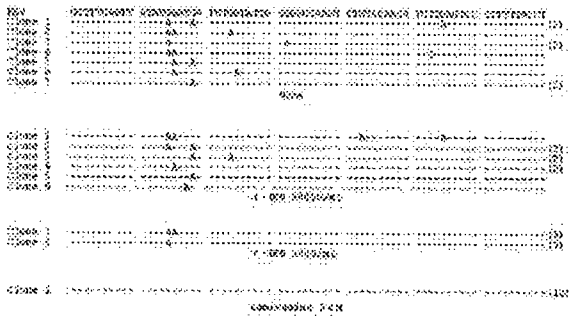

Fig. 6. Nucleotide sequence substitutions around YMDD motif of reverse transcriptase detected by PCR with PNA clamping after treating a HepG2 cell line (cell line 2 in Table 3). The nucleotide sequence of the transfected clone was used as a reference sequence (**top line**). Cells were treated with interferons and lamivudine as shown in Figs. 5 and 7, respectively.

noticeable increase in HepG2 cells by our detection method (C. Noguchi and K. Chayama, unpublished data). However, the method employed to detect hypermutation is not quantitative. Moreover, no antibody to detect APOBEC3G is available. Measurement of activity of this enzyme might be necessary to address this issue.

Because the patterns of hypermutations found in patients as well as cell lines are in agreement with strong dinucleotide preferences of a retroviral genome[31-35] edited by APOBEC3G,[7-9] we assume that hypermutations might also be induced by a similar enzyme. As pointed out by Turelli et al.,[20-29] another deaminase including APOBEC3F might be responsible for the generation of hypermutation. We actually detected the expression of deaminases in HepG2 cell lines. The expression levels of these deaminases are very low because they were detected by only two-stage PCR with one exception (only APOBEC3F was detected by a single-stage PCR).

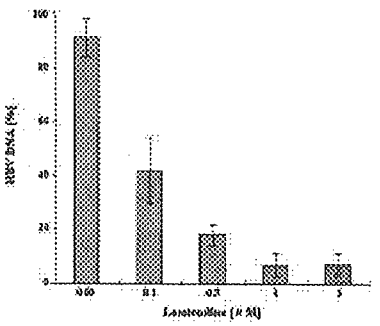

Fig. 7. Effects of lamivudine on production of HBV DNA by cell line 1. After 5 days of lamivudine treatment, the HBV DNA in core particles was immunoprecipitated and quantitated by real-time PCR. Data are mean ± SD of 4 independent experiments.

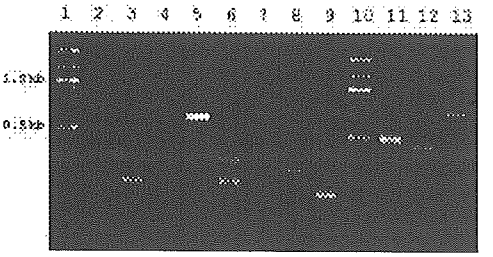

Fig. 8. Agarose gel electrophoresis of mRNAs of known deaminases amplified by reverse transcription-polymerase chain reaction. Lane 1: molecular weight size marker; lane 2: APOBEC1; lane 3: APOBEC2; lane 4: APOBEC3A; lane 5: APOBEC3B; lane 6: APOBEC3C; lane 7: APOBEC3D; lane 8: APOBEC3F; lane 9: APOBEC3G; lane 10: molecular weight size marker. Only mRNA of APOBEC3F was detected by one-stage PCR. To confirm the predictability of the assay, 3 negative mRNAs in Hep3G (APOBEC1, 3A and 3D) were amplified by using mRNAs from tissues known to express it. Lanes 11 and 12: APOBEC1 and APOBEC3A from the ileum; lane 13: APOBEC3D from the duodenum. All detected cDNAs were cloned, and nucleotide sequences were confirmed.

However, other possibilities should not be ignored. For example, some viral proteins might prevent such editing activity of deaminase by associating with this enzyme, as virion infectivity factor does in HIV-1–infected cells. Possibly the edited HBV genomes are degraded in liver cells rapidly by removal of the U residues by uracil DNA glycocylase followed by cellular nucleases.[36]

We found hypermutated genomes only in patients positive for eAb. The G to A nucleotide substitution of codon 28 of pre-core protein, which induces premature stop of this protein and basal core promoter mutations (A1762T/G1764A), might be related to the clearance of eAg.[28] Further studies should be conducted to investigate the relationship between G to A substitutions in these regions by deaminase(s), production of eAg, and replication efficacy of the virus.

A recent study showed that the amount of HBV DNA reduction occurs noncytopathologically through the action of cytokines, especially interferon alpha/beta and gamma.[37,38] We thus examined whether interferon can alter the occurrence of hypermutation. However, the results showed no increase in the number of hypermutation in HepG2–derived cell lines treated by interferon alpha and gamma (Fig. 6). Thus, the antiviral action of the mechanism responsible for G to A substitution in liver cells is likely to be independent of the action of interferon.

In conclusion, numerous innate intracellular defense systems exist, and the precise pathways of such systems are not fully understood. The role of editing of the HBV genome in such defense systems should be further investigated to understand the natural antiviral mechanisms and to develop an antiviral strategy against HBV.

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Suppression of Macrophage Infiltration Inhibits Activation of Hepatic Stellate Cells and Liver Fibrogenesis in Rats

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Background & Aims: Monocytes/macrophages infiltrate into injured livers. We tried to clarify their roles in inflammation and subsequent fibrogenesis by inhibiting their infiltration with a mutated form (7ND; 7 amino acids at the N-terminal were deleted) of monocyte chemoattractant protein 1, which may function as a dominant-negative mutant. *Methods:* Rats were injected via the tail vein with an adenovirus expressing either human 7ND (Ad7ND), a truncated type II transforming growth factor β receptor (AdTβ-TR), which works as a dominant-negative receptor, bacterial β-galactosidase (AdLacZ), or saline. Seven days later, the rats were treated with dimethylnitrosamine for 1–21 days. *Results:* Within 24 hours after a single dimethylnitrosamine injection, macrophages were observed in livers. With a 3-day dimethylnitrosamine treatment, activated hepatic stellate cells were detectable in livers in AdLacZ-, AdTβ-TR–, and saline-injected rats. In contrast, in the Ad7ND-treated rats, infiltration of macrophages was markedly reduced, and activated hepatic stellate cells were not detectable. After a 3-week dimethylnitrosamine treatment, fibrogenesis was almost completely inhibited, and activated hepatic stellate cells were hardly seen in livers in both Ad7ND- and AdTβ-TR–treated rats. *Conclusions:* Our results show that blockade of macrophage infiltration inhibits activation of hepatic stellate cells and leads to suppression of liver fibrogenesis. The presence of activated hepatic stellate cells in the initial phase after injury and its absence at a later phase in the AdTβ-TR–treated livers indicate that transforming growth factor β is not an activating factor for hepatic stellate cells, and this suggests that transforming growth factor β is required for the survival of activated hepatic stellate cells. Our study suggests that infiltrated macrophages may themselves produce an activating factor for hepatic stellate cells.

Inflammation is always accompanied by an infiltration by leukocytes,[1] a process that is thought to be regulated by chemotactic cytokines called *chemokines*.[1,2] Monocyte chemoattractant protein (MCP)-1, one of these chemokines, induces infiltration by monocytes/macrophages and lymphocytes[3] by binding to a specific receptor, CCR2.[1,2] In animal models of liver injury[4,5] and in patients with chronic hepatitis,[6,7] MCP-1 is detectable in both livers and serum. Injury-induced inflammation results in tissue remodeling or liver fibrosis. However, the actual roles performed by infiltrated monocytes/macrophages and MCP-1 in liver fibrogenesis are largely unknown.

During liver fibrogenesis, hepatic stellate cells (HSC) are activated to myofibroblast-like cells expressing α-actin. These activated HSC and myofibroblasts already existing in the portal field and around central veins may play a central role in fibrogenesis,[8] after which they produce extracellular matrix through the generation of various cytokines, including transforming growth factor (TGF)-β.[9] For fibrogenesis, HSC are considered to be the responsible cells, and TGF-β is one of the critical factors for fibrogenesis. In fact, when we inhibited the action of TGF-β by using a dominant-negative mutated receptor for TGF-β,[10] the activated HSC were markedly reduced in number, and fibrogenesis, as well as the progression of already-established fibrosis, was almost completely suppressed.[11–13] This shows the essential roles played by TGF-β and HSC in fibrotic remodeling after liver injury. However, the mechanism underlying the activation of HSC is not fully understood, although TGF-β has been believed to be an activating factor.[14]

In this study, to try to answer these questions, we introduced a mutated form of MCP-1 (7ND), which is

Abbreviations used in this paper: DMN, dimethylnitrosamine; ELISA, enzyme-linked immunosorbent assay; HSC, hepatic stellate cells; MCP, monocyte chemoattractant protein; MOI, multiplicity of infection; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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considered to inhibit the action of MCP-1 as a dominant-negative mutant,[15,16] into dimethylnitrosamine (DMN)-treated rats, an established model of liver fibrosis with a pathology closely resembling that of human cirrhosis.[17,18] Some rats were given a dominant-negative TGF-β receptor to eliminate signaling by TGF-β.[11,12] We compared these rats in terms of (1) infiltration by monocytes/macrophages and activation of HSC, both of which occur in the acute phase after injury, and (2) fibrotic changes in the chronic phase after injury. Although inhibition of MCP-1 and blockade of TGF-β each led to a marked suppression of liver fibrogenesis, we were interested to find that some responses in the initial phase after injury were quite different between these 2 groups. Our study indicates that TGF-β is not an activating factor for HSC and suggests that infiltrated monocytes/macrophages may produce the activating factor(s).

Materials and Methods

Preparation of Adenoviruses

Replication-defective $E1^-$ and $E3^-$ adenoviral vectors expressing an amino-terminal deletion mutant of human MCP-1 (Ad7ND) with a FLAG epitope tag in its carboxyl-terminal (complementary DNA, a generous gift from Dr. B. Rollins, Harvard University),[15,16] a truncated human TGF-β type II receptor (AdTβ-TR),[10–12] or bacterial β-galactosidase (AdLacZ)[19] under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter[20] were prepared as previously described.[21]

Detection of Mutated Human Monocyte Chemoattractant Protein 1 (7ND) and Rat Wild-Type Monocyte Chemoattractant Protein 1

COS cells were infected with either Ad7ND (multiplicity of infection [MOI] of 1, 10, and 100) or AdLacZ (MOI of 10), as previously described.[10] One day after infection, the medium was replaced with serum-free medium, and cells were incubated for a further 24 hours. A mutant MCP-1 (7ND) secreted into culture media was analyzed by Western blotting by using monoclonal antibodies against either FLAG (Abcam, Cambridge, UK) or human MCP-1 (Sanbio, 5400 AM Uden, The Netherlands), as previously described.[13]

7ND and rat MCP-1 were also detectable by enzyme-linked immunosorbent assay (ELISA). Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000*g* for 30 minutes. 7ND and rat MCP-1 were measured in the supernatant of liver homogenates and in sera from rats by using a human MCP-1 ELISA kit (Biosource, Camarillo, CA) and a rat kit (Biosource), respectively, according to the manufacturer's instructions. These ELISA kits are species specific, and cross-reaction between human and rat MCP-1 is less than 5%. In fact, no human MCP-1 protein was detectable in samples from either intact or AdLacZ-infected rats (data not shown).

Animal Models

All animals were treated under protocols approved by the institutional animal care committees, and the experiment was performed under both the institutional guidelines for animal experiments and by the Law (No. 105) and Notification (No. 6) of the Japanese government. Male Sprague–Dawley rats, 10 weeks old and weighing approximately 350 g, were given a single infusion of 0.5 mL of Ad7ND, AdTβ-TR, AdLacZ (2×10^9 plaque-forming units per milliliter), or saline via the tail vein, as previously reported.[12] By this method, virtually all cells in the liver were infected and expressed the introduced molecule.[11,12] Seven days later, rats were given an intraperitoneal injection of DMN (10 μg/g body weight; Wako, Osaka, Japan) either once or at the indicated times (3 consecutive daily injections or 3 consecutive daily injections and 4 days off per week for 3 weeks), as previously reported.[11–13] After DMN treatment, blood was collected, and the rats were killed. Biochemical parameters were measured by using standard methods. The liver was either fixed with 4% buffered paraformaldehyde for histological examination or frozen immediately in liquid nitrogen for the extraction of hydroxyproline, the content of which was measured as described elsewhere.[22]

Histological Examination

Liver sections were stained with hematoxylin or Masson trichrome or subjected to immunohistostaining by using antibodies against either CD68 (ED-1; Serotec, Raleigh, NC) or α-actin (Dako, Tokyo, Japan). Immunoreactive materials were visualized by using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) and diaminobenzidine. Macrophages (CD68-positive cells) and lymphocytes were counted by a technician blinded to the treatment regimen. Four random high-power (200×) fields from each section were examined. As negative controls, immunohistostaining was performed without the first antibodies.

Determination of Hepatic Stellate Cells in Apoptosis

Fragmented DNA in apoptotic cells in liver sections was stained with diaminobenzidine (dark brown) by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique by using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Then, the sections were double-stained against α-actin and visualized with the aid of 3-amino-9-ethyl carbazole liquid substrate chromogen (red; Dako). As negative controls, the TUNEL reaction mixture was used without terminal transferase.

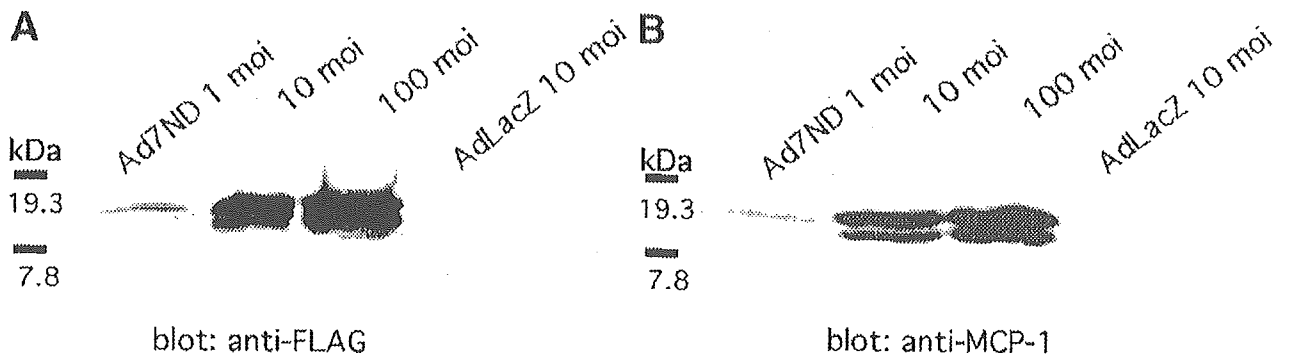

Figure 1. A mutated form of MCP-1 (7ND) is secreted from cells infected with Ad7ND. COS cells were infected either with Ad7ND or with AdLacZ at the indicated MOI. After 48 hours, the culture media were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and analyzed by Western blotting by using antibodies against either (*A*) FLAG or (*B*) human MCP-1. Molecular markers are in kilodaltons.

Statistical Analysis

Statistical analysis was performed by 1-way analysis of variance followed by Scheffé's test. $P < .05$ was considered significant.

Results

A Mutant Monocyte Chemoattractant Protein 1, 7ND, Was Secreted From Ad7ND-Infected Cells and Detected in the Serum and Liver of Ad7ND-Infected Rats

In the culture medium from Ad7ND-infected COS cells, 7ND was readily detectable in an MOI-dependent manner, as assessed by Western blotting analysis (Figure 1). Human 7ND and endogenous rat MCP-1 proteins were measured in sera (Figure 2*A*) and liver extracts (Figure 2*B*) from rats infected with Ad7ND. Seven days after gene transfer, a 3-week DMN treatment was begun. It is interesting to note that the amount of rat MCP-1 was not significantly changed by DMN treatment in either serum or liver. 7ND reached a peak on the seventh day after gene transfer and then declined gradually; however, the values were much higher than those obtained for rat MCP-1 in most time periods under DMN injury.

Dimethylnitrosamine-Induced Infiltration by Macrophages and Lymphocytes and Activation of Hepatic Stellate Cells Were Both Suppressed in Ad7ND-Treated Livers

Rats were infused via the tail vein with either saline or an adenovirus expressing 1 of the following:

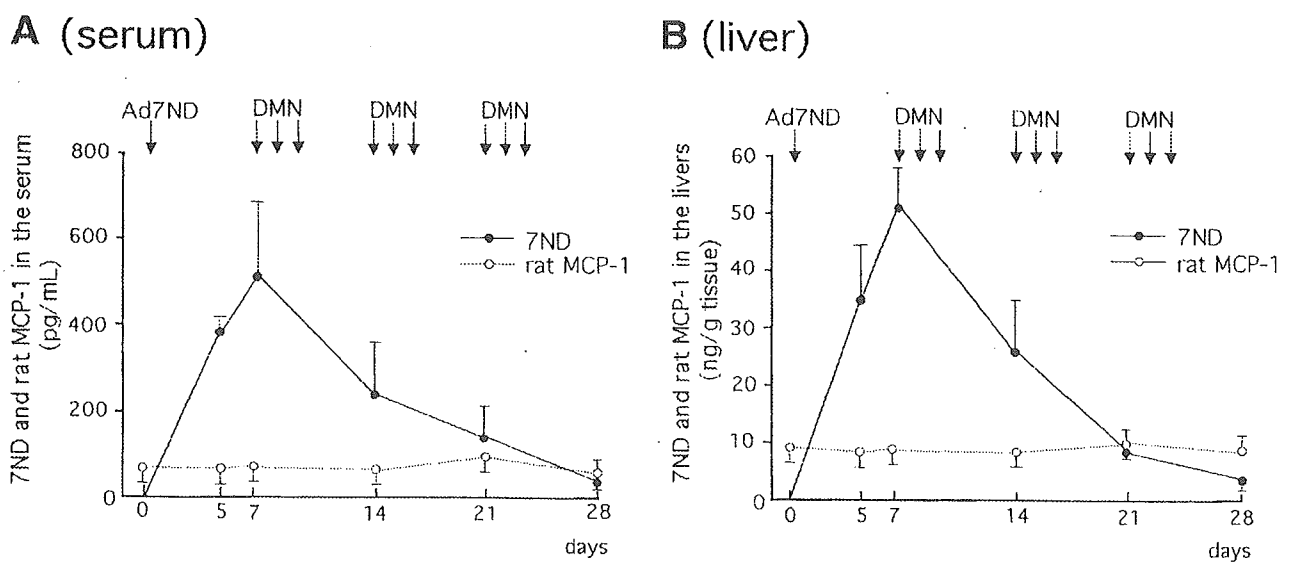

Figure 2. Amounts of human 7ND and rat MCP-1 in the sera (*A*) and livers (*B*) of DMN-injured rats. Rats were given a single infusion of Ad7ND (or saline infusion) via the tail vein. Seven days later, rats were subjected to a 3-week DMN treatment (shown as arrows). Rats were killed 5, 7 (just before the initiation of DMN treatment), 14, 21, and 28 days after Ad7ND injection. Means ± SD ($n = 4$) are shown.

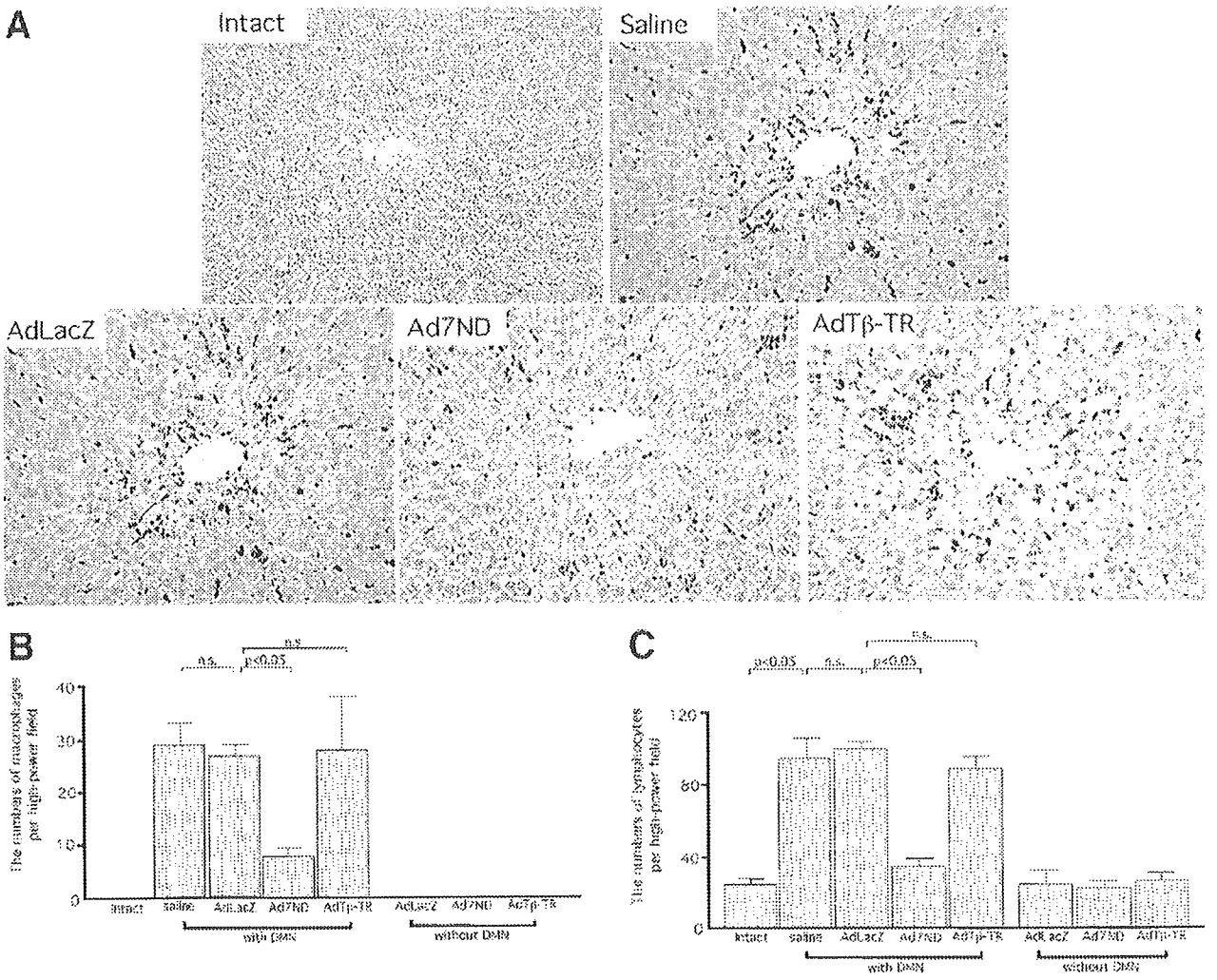

Figure 3. Infiltration of macrophages and lymphocytes into DMN-treated livers. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdTβ-TR via the tail vein. Seven days later, some rats were administered DMN once (shown as *with DMN*) and some rats were not administered DMN (shown as *without DMN*; histology not shown). Twenty-four hours after the DMN challenge, they were killed, and liver sections were histologically examined either by immunohistostaining against CD68, to detect macrophages (*A*), or by hematoxylin staining for lymphocytes (not shown; original magnification, 200×). Similar histology was seen in all 4 rats in each group. The numbers of (*B*) macrophages and (*C*) lymphocytes were semiquantitated (see Materials and Methods). Four fields in each of 4 rats (a total of 16 fields in each group) were examined, and the number of cells per high-power field is shown as mean ± SD. n.s., statistically not significant. Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*).

β-galactosidase (AdLacZ), a truncated TGF-β receptor (AdTβ-TR), or a mutated MCP-1 (Ad7ND). Seven days later (when the expression of the introduced molecules had reached a submaximal level), DMN was given. One day after a single injection of DMN, we analyzed liver sections by hematoxylin staining and immunohistostaining against CD68, which is a specific marker for macrophages. Macrophages were detectable in the centrilobular area of the livers of AdLacZ-infected, AdTβ-TR–infected, or saline-injected rats: there were no differences among these 3 groups. However, macrophages were greatly reduced in Ad7ND-treated livers (Figure 3*A*). The numbers of CD68-positive cells (per high-power field) were 29 ± 3.5 in saline-treated livers, 27.5 ± 2.1 in AdLacZ-treated livers, 27.5 ± 11.4 in AdTβ-TR–treated livers, and only 7.1 ± 1.2 in Ad7ND-treated livers (Figure 3*B*). Similarly, the numbers of lymphocytes (histology not shown) were 98 ± 7.5 in saline-treated livers, 101 ± 2.5 in AdLacZ-treated livers, 93 ± 5.5 in AdTβ-TR–treated livers, and only 40 ± 3.5 per high-power field in Ad7ND-treated livers (Figure 3*C*). Without DMN treatment, neither macrophages nor lymphocytes (histology not shown) were increased in the livers of AdLacZ-infected, AdTβ-TR–infected, and

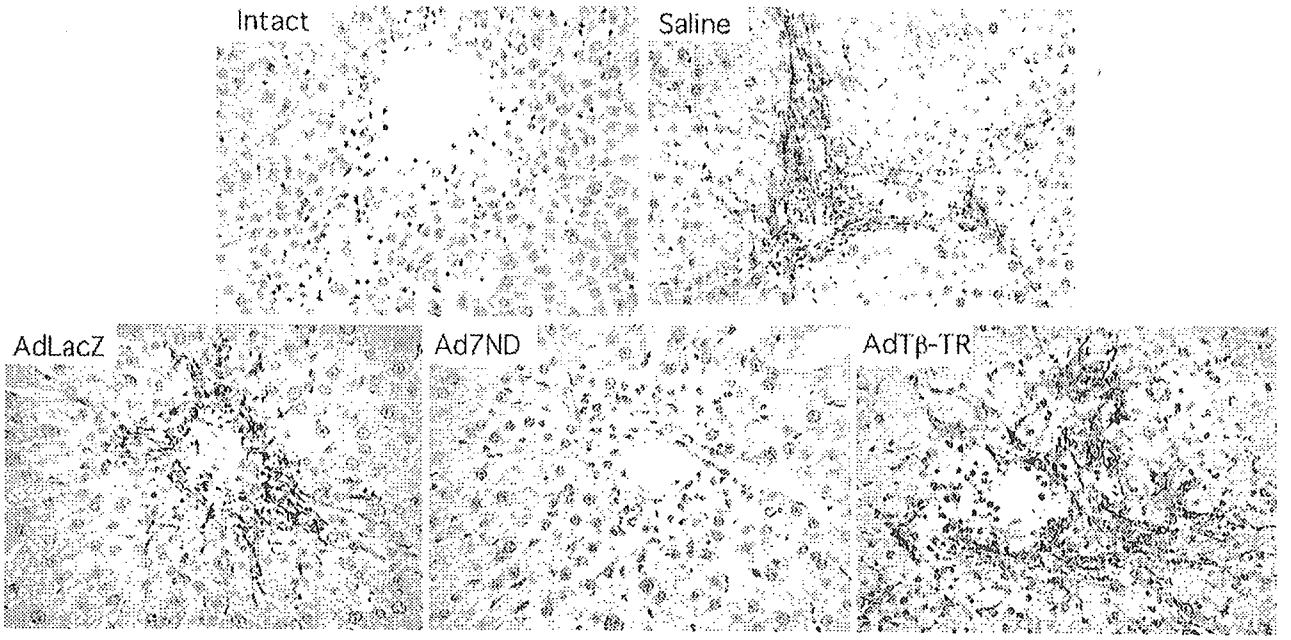

Figure 4. α-Actin–positive cells in DMN-treated livers. Rats were treated with either an adenovirus or saline as described in the legend to Figure 3 and then subjected to DMN for 3 consecutive days. One day after the last DMN injection (the fourth day), livers were examined by immunohistostaining against α-actin (original magnification, 200×). Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*). Similar histology was seen in all 4 rats in each group.

Ad7ND-infected livers compared with intact livers (subjected to no injection of either saline or adenovirus and no DMN treatment; Figure 3*B* and *C*).

Next, after a 3-day DMN treatment, we examined livers for α-actin–positive cells (a marker of activated HSC). They were readily detectable, not only in AdLacZ- or saline-treated, but also in AdTβ-TR–treated livers. In contrast, we could see none in the Ad7ND-treated livers (Figure 4).

Inhibition of Macrophage Infiltration or of Transforming Growth Factor β Signaling Markedly Suppresses Liver Fibrogenesis and Preserves Liver Function

After a 3-week DMN treatment, the hydroxyproline content of livers was measured as a quantitative evaluation of fibrosis (Figure 5). The hydroxyproline contents in the livers of both AdLacZ- and saline-treated rats were approximately 3-fold higher than in intact livers, as previously observed.[10–12] In contrast, in the Ad7ND-treated and AdTβ-TR–treated livers, the hydroxyproline content remained close to the level seen in intact livers.

After the DMN treatment, the serum levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin were all increased, and both the body and liver weights were decreased, probably because of liver dysfunction. However, these values were preserved or better maintained in the Ad7ND-treated or AdTβ-TR–treated groups (Table 1).

After a 3-week DMN treatment, we analyzed liver histology both by Masson trichrome staining and by immunohistostaining against α-actin. In accordance with the data on hydroxyproline content (Figure 5), both Ad7ND-treated and AdTβ-TR–treated livers showed a fibrotic area that was markedly smaller than that seen in

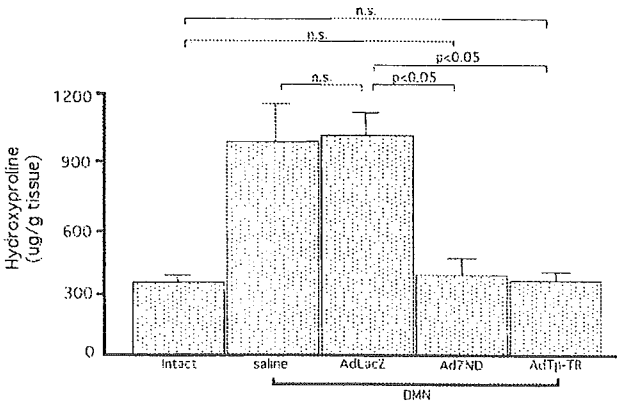

Figure 5. Hydroxyproline content of DMN-treated livers. Rats were treated with either adenovirus or saline as described in the legend to Figure 3 and then subjected to a 3-week DMN treatment. Hydroxyproline content of livers is shown as mean ± SD. Three samples from each of 4 rats were analyzed for each group. n.s., statistically not significant. Rats never treated with adenovirus or DMN were also analyzed (shown as *intact*).

Table 1. Serum Hepatobiliary Parameters and Body and Liver Weights

Variable	Total bilirubin (mg/mL)	AST (IU/mL)	ALT (IU/mL)	Body weight (g)	Liver weight (g)
Intact	0.2 ± 0.1	68 ± 16	40 ± 9	350 ± 20	3.5 ± 0.1
AdLacZ	0.2 ± 0.1	71 ± 11	39 ± 7	340 ± 20	3.6 ± 0.1
Saline + DMN	0.7 ± 0.6	495 ± 103	245 ± 88	290 ± 20	2.4 ± 0.4
AdLacZ + DMN	0.8 ± 0.7	525 ± 149	232 ± 97	290 ± 30	2.3 ± 0.5
Ad7ND + DMN	0.3 ± 0.1[a]	134 ± 16[a]	69 ± 7[a]	350 ± 10[a]	3.4 ± 0.1[a]
AdTβ − TR + DMN	0.4 ± 0.1[a]	222 ± 84[a]	69 ± 25[a]	350 ± 10[a]	3.5 ± 0.1[a]

NOTE. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdTβ-TR via the tail vein. Seven days later, a 3-week DMN treatment was given to some rats (shown as +DMN). After a 3-week DMN treatment, blood was collected, and body and liver weights were measured. Serum total bilirubin, AST, and ALT and body and liver weights are shown as mean ± SE (n = 4). Rats never subjected to adenovirus infection or treated with DMN were also measured (shown as Intact).
AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[a]$P < .05$ vs. AdLacZ + DMN.

the AdLacZ- and saline-injected rats, and α-actin–positive cells were almost undetectable (Figure 6).

In the AdTβ-TR–treated livers, α-actin–positive cells were readily detectable after the initial 3-day DMN treatment (Figure 4). We assumed that activated HSC disappeared through apoptosis under conditions in which the action of TGF-β was suppressed. We therefore performed TUNEL staining on the fourth day after starting DMN treatment. TUNEL-positive cells were increased in the AdTβ-TR–treated livers; however, no such apoptotic cells were observed in the AdLacZ- or saline-injected livers (Figure 7*A*). Immunohistostaining against α-actin confirmed that the TUNEL-positive cells in the AdTβ-TR–treated livers (Figure 7*A*) were indeed α-actin positive (Figure 7*B*).

Discussion

Inflammation induces infiltration by leukocytes and monocytes/macrophages into inflamed tissues.[1] Tissue remodeling or fibrosis then follows the inflammation. MCP-1, one of the CC chemokines, attracts monocytes/macrophages bearing CCR2.[1–3] In this study, the roles of such macrophages in injury-induced liver fibrogenesis were investigated by overexpressing a mutated MCP-1 (7ND), which is reported to suppress the actions of MCP-1.[15,23–25] In the Ad7ND-treated rats, DMN-induced infiltration by macrophages and lymphocytes into injured livers was markedly suppressed (Figure 3), the activation of HSC was eliminated (Figure 4), and liver fibrogenesis was greatly prevented (Figures 5 and 6). The cellular infiltration and activation of HSC observed immediately after infliction of the injury were similar between the AdTβ-TR–treated livers and the controls (saline-infused or AdLacZ-infected rats; Figures 3 and 4). Our study shows that infiltrated macrophages are critical for HSC activation and subsequent fibrogenesis and, importantly, that TGF-β is not an activating factor for HSC. It is suggested that the infiltrated macrophages

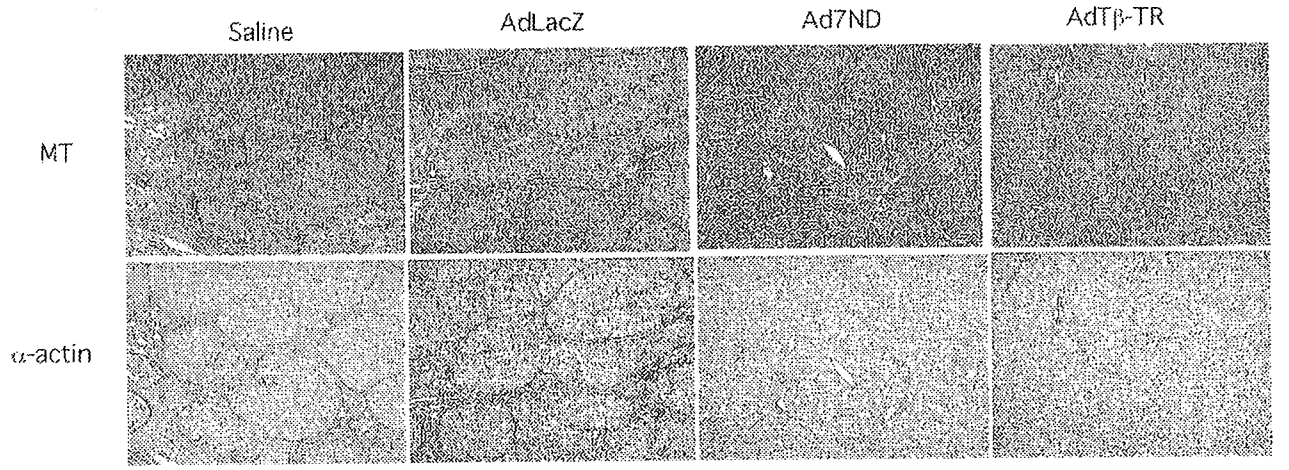

Figure 6. Histology of livers after a 3-week DMN treatment. Rats were treated as described in the legend to Figure 5. Liver sections were histologically examined with the aid of Masson trichrome staining (MT) or by immunohistostaining against α-actin (original magnification, 100×). Similar histology was seen in all 4 rats in each group.

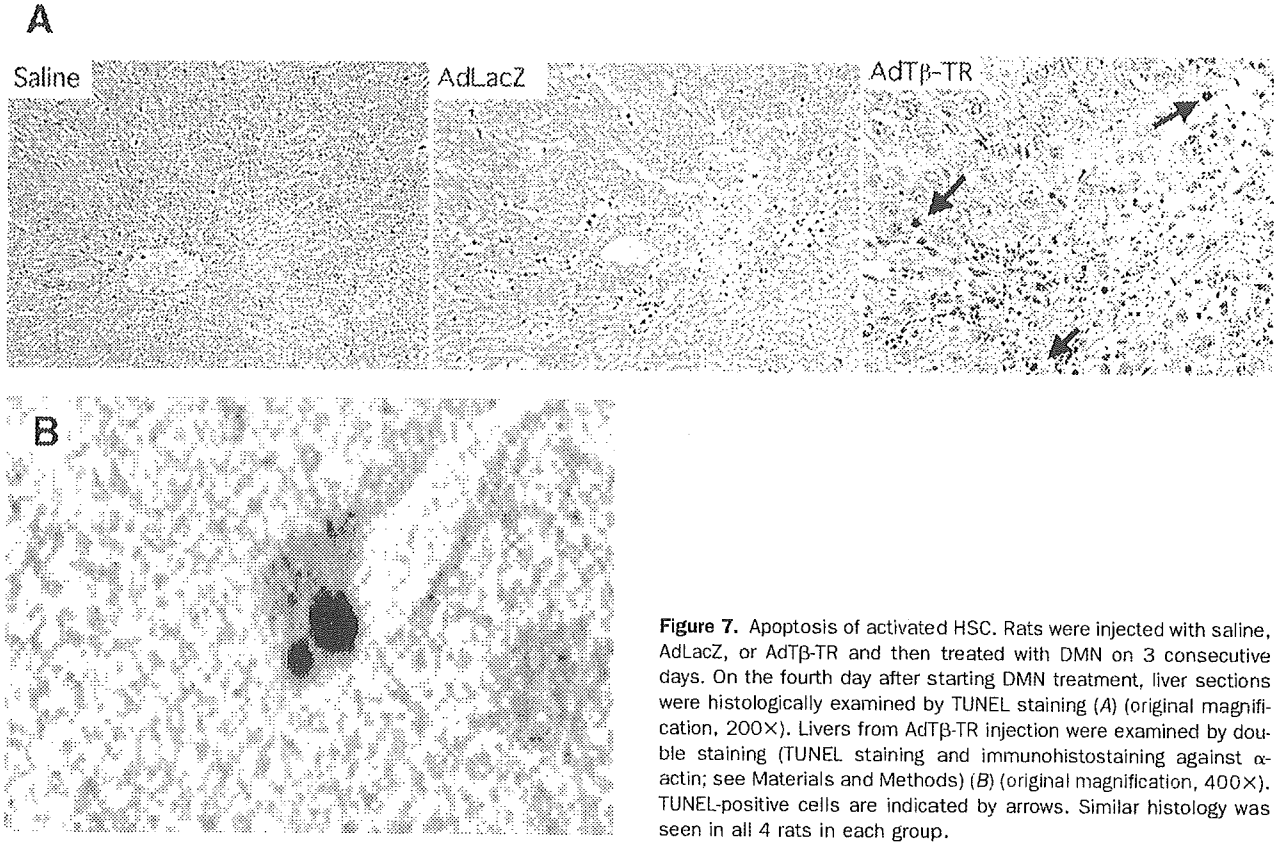

Figure 7. Apoptosis of activated HSC. Rats were injected with saline, AdLacZ, or AdTβ-TR and then treated with DMN on 3 consecutive days. On the fourth day after starting DMN treatment, liver sections were histologically examined by TUNEL staining (*A*) (original magnification, 200×). Livers from AdTβ-TR injection were examined by double staining (TUNEL staining and immunohistostaining against α-actin; see Materials and Methods) (*B*) (original magnification, 400×). TUNEL-positive cells are indicated by arrows. Similar histology was seen in all 4 rats in each group.

may themselves secrete an activating factor or factors for HSC.

We have previously shown that anti–TGF-β intervention inhibits liver fibrogenesis[11,13] and its progression.[12] In this study, we found that suppression of infiltration by macrophages and lymphocytes through overexpression of 7ND led to a powerful suppression of liver fibrogenesis to a similar degree as blockade of TGF-β but that the underlying mechanisms seem to be different. Activation of HSC in the initial stage immediately after injury was already eliminated in the Ad7ND-treated livers (Figure 4). Probably because HSC activation was inhibited, the subsequent progress toward fibrosis was suppressed in the Ad7ND-treated livers, thus supporting the idea that activation of HSC is the initial and critical event that leads to liver fibrosis. It has been considered for a long time that TGF-β is the HSC-activating factor (or at least one of the activating factors).[14] However, our study clearly shows for the first time that TGF-β is not the HSC-activating factor, because a substantial number of activated HSC were present in the AdTβ-TR–treated livers (Figure 4); indeed, the numbers of activated HSC were the same among saline-treated, AdLacZ-treated, and AdTβ-TR–treated livers. We confirmed previously that virtually all liver cells are infected with an adenovirus when one is administered to rats with intact livers,[11,13] so the possibility can be excluded that all of these activated HSC were uninfected with AdTβ-TR. Although substantial numbers of activated HSC were seen after a 3-day DMN treatment, most disappeared during the next 2 weeks of DMN treatment (Figure 6). The activated HSC are probably eliminated through apoptosis under conditions in which TGF-β signaling is inhibited. Indeed, we showed that in the AdTβ-TR–treated livers, but not in the AdLacZ- or saline-injected ones, activated HSC were in apoptosis (Figure 7). Saile et al[26] reported that HSC undergo CD95-mediated spontaneous apoptosis when they are activated, and TGF-β inhibits CD95-agonistic antibody-induced apoptosis of activated HSC in culture.[27] On the basis of these reported findings and our present study, it is likely that TGF-β is required for the activated HSC to survive. Consequently, fibrogenesis was markedly inhibited in the AdTβ-TR–treated livers despite activation of HSC in the initial stage after injury. To judge from our findings, anti–TGF-β intervention ought to be superior to anti–MCP-1 therapy for treating liver cirrhosis patients, most of whom already

have some degree of fibrosis or injury. This issue is now under further investigation in our laboratory.

Marra et al[28] reported that MCP-1 enhances the migration of HSC in culture. Moreover, it has been reported that HSC themselves produce MCP-1[29,30] and that TGF-β induces the secretion of MCP-1.[29] Together with our present study, it is likely that MCP-1 stimulates liver fibrogenesis by 2 mechanisms: (1) MCP-1 induces macrophage infiltration, and macrophages secrete an activating factor(s) for HSC; (2) MCP-1 acts directly on activated HSC to modulate their function. In favor of this notion, it has been reported that in cultured skin fibroblasts, MCP-1 increases the gene expressions of α_1 (I) procollagen and TGF-β[31] and of matrix metalloproteinase 1 and 2 (and of their inhibitor, tissue inhibitor of metalloproteinase 1)[32] and that MCP-1 stimulates the proliferation of cultured vascular smooth muscle cells.[33] Collectively, these observations suggest that MCP-1 secreted from macrophages and from HSC themselves may facilitate the production of TGF-β, as well as of matrix metalloproteinases and their inhibitors, thereby enhancing inflammation and tissue remodeling (fibrogenesis).

We expressed 7ND in livers expecting that it would inhibit MCP-1 as a dominant-negative mutant.[16] It has been reported that a 75:1 molar ratio of 7ND/wild-type MCP-1 is needed for a 50% inhibition of monocyte chemotaxis in vitro.[16] In our setting, the amount of 7ND was substantially higher than that of rat MCP-1 in both sera and livers for at least 2–2.5 weeks under DMN treatment (Figure 2). We have not yet determined how much 7ND is required to inhibit the actions of MCP-1 in vivo, specifically in the case of the DMN-injured rat liver. Thus, it is not certain whether the actions of MCP-1 were indeed inhibited in our experiments or whether the observed inhibition of macrophage infiltration was indeed achieved via a suppression of MCP-1 by 7ND. We would like to add that numerous reports (13, to our knowledge) have been published in which the same 7ND construct as that used in this study was introduced (either by direct injection or by electroporation with an expression plasmid) into various animal models, and suppression of macrophage infiltration and some biological effects were seen in every one of these reports. In 3 of these 13 studies, both 7ND and endogenous MCP-1 proteins in serum were measured, and the values obtained (7ND/MCP-1) were 220/71 pg/mL,[23] 226/85 pg/mL,[24] and 124/92 pg/mL[25] (all in mice). We detected a peak value of 528 ± 182 pg/mL for 7ND and 62 ± 12 pg/mL for endogenous rat MCP-1. Both this peak value for 7ND and the ratio between 7ND and endogenous MCP-1 are the highest among the values reported in the literature so far.

In summary, we have shown that the macrophages that infiltrate into livers immediately after an initial injury are critical both for HSC activation and for the subsequent fibrogenesis, and we also showed that TGF-β, which is required for activated HSC to survive, is not an activating factor for HSC (at least in this situation). Macrophages may themselves secrete an activating factor(s) for HSC.

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Review

Antiviral therapy for chronic hepatitis C: past, present, and future

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Antiviral therapy for chronic hepatitis C has dramatically advanced since the discovery of the hepatitis C virus (HCV) in 1989 and the introduction of interferon (IFN) monotherapy in the early 1990s. The current standard therapy uses a combination of pegylated IFN and ribavirin. The duration of therapy and response to therapy are HCV genotype-specific. Genotype 1 patients require 48 weeks of the combination therapy for 50% successful viral elimination, while genotype 2 patients require 24 weeks of therapy for 80% or 90% viral elimination. Early viral kinetics after the initiation of therapy is a useful predictor of the sustained virologic response (SVR), which is formally determined at 24 weeks after completion of the treatment. For example, an early virologic response, which is determined by a 2-log reduction of HCV RNA or viral elimination at 12 weeks after the initiation of therapy, is a strong negative predictor of SVR in genotype 1 patients. In contrast, a rapid virologic response of HCV RNA-negative at 4 weeks after the initiation of therapy identifies genotype 2 "super-responders," who may require a shorter period of therapy. Adherence to therapy is one of the most important factors for successful viral clearance. Hematopoietic growth factors such as epoetin and granulocyte-colony stimulating factor help reduce therapy-mediated cytopenia and improve patient compliance, thereby leading to better viral clearance. New types of anti-HCV agents such as HCV protease and polymerase inhibitors are needed for those patients that do not respond to combination therapy.

Introduction

In 1989, the hepatitis C virus (HCV) was discovered in the United States to be the causative agent of posttransfusion non-A, non-B hepatitis by Chiron Corporation (Emeryville, CA, USA).[1] By this discovery, HCV was revealed to be the cause of many hepatic diseases of previously unknown origin. HCV is closely associated with hepatocellular carcinogenesis and death due to chronic liver disease. Epidemiologically speaking, it is estimated that 1.7 million people in Japan and 170 million people worldwide are infected with HCV.[2] Many cases are asymptomatic and result in overt hepatic disease, manifested as hepatic cirrhosis or cancer, only following 20 to 30 years of persistent infection. Thus, HCV infection is of significant concern in terms of public health.

Spontaneous elimination of HCV occurs in approximately 30% of HCV-infected patients within 6 months after infection. However, after this period of time, viral elimination is very rare, with an annual rate of only about 0.2%. Persistent inflammation associated with HCV causes hepatic fibrosis, and as the stage of fibrosis progresses, the risk of cancer increases; annual rates of hepatocarcinogenesis are 0.5% for patients with modest fibrosis and 8% for those with liver cirrhosis.

HCV-associated, progressive hepatic disease can be directly inhibited by interferon (IFN), which is currently the only drug that can eradicate HCV. This review traces the progress of IFN-based therapy for hepatitis C since its introduction and provides a brief overview of the future of HCV treatment.

Introduction of IFN therapy

IFN therapy for hepatitis C dates from 1986, when Hoofnagle et al.[3] reported the normalization of serum alanine aminotransferase (ALT) levels following administration of recombinant human IFNα to patients with non-A, non-B hepatitis. In other words, IFN was shown to be biochemically effective as an anti-inflammatory agent before the discovery of HCV.[4,5]

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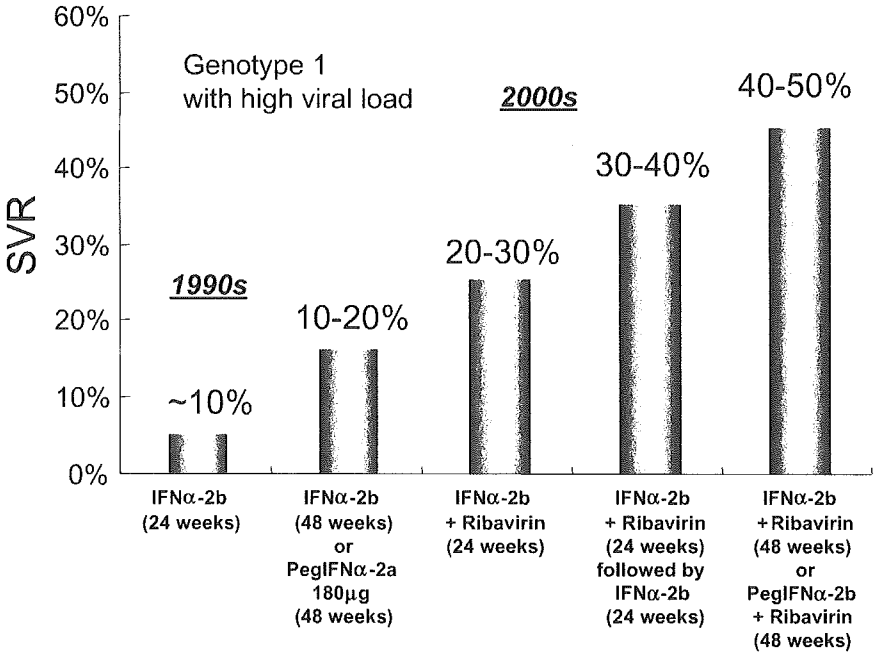

Fig. 1. Milestones of interferon (*IFN*)-based therapy for chronic hepatitis C. Progress in sustained viral clearance for a difficult to treat patient group, with genotype 1 and a high viral load, from the early 1990s. *SVR*, sustained virologic response; *PegIFN*, pegylated interferon

Later, the introduction of a virus identification method using a polymerase chain reaction (PCR) assay revealed cases in which patients became HCV-PCR negative following IFN administration.[6] The normalization of serum ALT levels is associated with viral eradication, with the exception of a few cases. The discovery of these biochemical and virological effects prompted approval of clinical use of IFN against hepatitis C in the United States in 1991 and in Japan in 1992.

The therapeutic responses to IFN can be classified as sustained virologic response (SVR), relapse, or nonresponse. SVR means complete elimination of HCV, which is defined as the loss of detectable HCV RNA during therapy and its continued absence for at least 6 months after the termination of therapy. Relapse is defined as being HCV-negative at the end of IFN treatment but HCV-positive within 6 months after the termination of therapy. Nonresponse is defined as the absence of a HCV-negative condition even with IFN administration. Initial studies showed that after 6 months of 6MU IFN administration to patients with chronic hepatitis C, SVR, relapse, and nonresponse were each observed in one-third of the patients. Subsequent studies revealed that the antiviral effect is determined mainly by viral factors, namely the viral load and the viral genotype.[7,8] Genotypes 1a and 1b are more resistant to IFN therapy than genotypes 2a and 2b, and patients with a high viral load are less likely to respond to IFN than those with a lower viral load. A subgroup analysis of patients treated with IFN monotherapy showed that the SVR rate in genotype 1 patients with a high viral load, accounting for approximately 60% of patients with hepatitis C in Japan, was only 5%. How to improve the therapeutic effect in these patients is the greatest problem for future research and development of IFN therapy (Fig. 1).

Progress of IFN-based therapy

For such resistant cases (patients with genotype 1 and high viral load), extended administration to optimize the total dose of IFN, the introduction of pegylated IFN (PEG-IFN) and coadministration with ribavirin have been used to substantially improve treatment over the past 10 years.

Optimization of the total dose of IFN: extended administration

Two means of increasing the total dose of IFN in resistant cases have been investigated: increasing the dose and extending the administration period. In Japan, patients had usually been given 6MU IFN three times a week for 6 months. Higher doses did not correlate with an increased SVR rate, partly because of the increased incidence of adverse effects and reduced patient compliance. However, extending the administration period proved effective for raising the SVR rate. Kasahara et al.[9] showed that 12 months of administration clearly

increased the SVR rate in genotype 1 patients, compared with 6 months of administration. However, in genotype 2 patients, there was no significant difference between 6 and 12 months of administration. The standard dose of IFN used in Europe and the United States, based on early clinical studies, has been 3 MU three times a week, with the result that European and U.S. therapeutic results after 6 months of IFN monotherapy are generally lower than those in Japan.[10,11] In Europe and the United States, the superiority of the 6-MU dose over a 3-MU dose has been shown by subsequent controlled studies, and many other clinical studies have shown the superiority of 12 months of therapy over 6 months.[12–14] The SVR rate for genotype 1 patients with a high viral load improved with IFN therapy of extended duration, shown first for IFN monotherapy and later for the combination of IFN with ribavirin.[15–17]

Based on these findings, administration of IFN for 12 months was approved early in Europe and the United States. In Japan, the 6-month limit for IFN therapy was removed in 2002, and self-injection of IFN was approved in 2005. These measures make it easier for patients to undergo long-term treatment.

Development of IFN preparations: introduction of PEG-IFN

The type I IFNs include IFNα, IFNβ, IFNω, and IFNλ, all of which share cell-surface receptor and intracellular pathways of action. IFN agents are used in various preparations. In the United States, recombinant IFNα-2b and IFNα-2a were initially approved. In Japan, in addition to these two preparations, natural IFNα and IFNβ can be used. These conventional preparations are considered to be of equal efficacy, although a few differences in the incidences of neutralizing antibodies and adverse effects have been noted.[18] Subsequently, a special agent, consensus IFN, was developed and put into clinical use.[19] It was designed by selecting the most frequently occurring amino acid at each site of the amino acid sequences of 13 known IFNα subtypes. Consensus IFN is considered to have a potent antiviral effect in genotype 1 patients with a high viral load, but it is still considered to be a conventional IFN agent.

Revolutionary progress in the development of IFN agents was recorded with the development of PEG-IFN and its introduction to clinical use. Pegylation is defined as modification of a drug by the addition of an artificial polymer, polyethylene glycol (PEG), for the purpose of delaying drug elimination, lowering its antigenicity, and modifying the drug's effect. Conventional IFN agents, with approximately 8-h elimination half-lives, require a dosing interval of 1 or 2 days to maintain an effective blood concentration.[20,21] The most beneficial effect of PEG-IFN is that it delays drug elimination, making it possible to maintain a stable blood concentration with once-weekly administration.[22] Currently, two PEG-IFN preparations are available: recombinant IFNα-2a and IFNα-2b, which are covalently bound to 40-kDa PEG and 12-kDa PEG, respectively. Both are thought to have about equal efficacy, but they have not been compared in clinical trials.

European and U.S. controlled studies have shown that PEG-IFN agents are generally more effective, both in monotherapy[23–25] and in combination with ribavirin, than conventional IFN agents.[26,27] In Japan, clinical studies have shown that PEG-IFN agents are not inferior to conventional IFN agents. However, no study has shown PEG-IFN agents to be significantly superior with respect to SVR, partly, perhaps, because the usual dose of control IFN agents used in Europe and the United States is 3 MU, which is less than that used in Japan. In sum, PEG-IFN is at least equivalent to conventional IFN in effectiveness, and it appears to be highly tolerable because it can be administered just once a week.

The adverse effects of IFN are classified into two types: those that occur soon after the start of administration, and those that manifest during long-term administration.[28] The former type includes flu-like symptoms, such as a high fever, headache, and myalgia, and abnormal blood test results such as thrombocytopenia and leukopenia. Effects seen with long-term administration include a wide variety of symptoms, such as pruritus, alopecia, fundal hemorrhage, depression, thyroid dysfunction, diabetes mellitus, pulmonary fibrosis, and cardiac arrhythmia. Adverse effects of PEG-IFN are similar to those of conventional IFN and are characterized by mild influenza-like symptoms during the early stage of administration and comparatively severe cytopenia. The occurrence of acute thrombocytopenia in the late stage of administration of PEG-IFNα-2a has also been noted. More caution is needed with respect to the occurrence of adverse effects of PEG-IFN owing to its delayed clearance.

Combination therapy: introduction of ribavirin

Ribavirin, developed in 1972, is a synthetic nucleic acid analog with a purine skeleton. It has antiviral activity in vitro to a wide variety of RNA and DNA viruses, and it is orally administered. Ribavirin has not been approved in Japan as an antiviral agent for monotherapy, but it has been approved in Europe and the United States for various viral diseases, such as severe respiratory syncytial virus infection in children. Its antiviral effect against HCV has not been proved by studies on monotherapy for hepatitis C.[29] In 1998, however, the combination of ribavirin with IFN was reported to have achieved a significantly higher SVR rate compared with IFN

monotherapy.[15,16,30,31] These reports were followed by large-scale clinical studies in Europe and United States[26,27] showing that a combination of PEG-IFN and ribavirin produces better results than one of IFN and ribavirin. With both combinations, 48 weeks of administration to genotype 1 patients achieved a significantly higher SVR rate than 24 weeks of administration.[15,16,32] For other patients, no significant difference was seen between groups receiving 24 or 48 weeks of therapy, and the 24-week administration period was reported to be sufficiently effective.

In Japan, a 48-week, multicenter, randomized, controlled study[33] was conducted on combinations of 6 MU IFNα-2b with ribavirin and 1.5 μg/kg PEG-IFNα-2b with ribavirin administered to genotype 1b patients with a high load of HCV-RNA, determined to be 100 KIU/ml or higher using Amplicor (by the original PCR method). Oral doses of ribavirin administered were 600 mg/day for patients weighing less than 60 kg, 800 mg/day for those weighing at least 60 kg but less than 80 kg, and 1000 mg/day for those weighing 80 kg or more. IFNα-2b was administered six times a week for the first 2 weeks and three times a week for the following 46 weeks, while PEG-IFNα-2b was administered once a week. The results for 506 patients indicated high rates of viral elimination by both therapies. The combination of PEG-IFNα-2b plus ribavirin and that of IFNα-2b and ribavirin achieved SVR in 121/254 patients (47.6%) and 113/252 patients (44.8%), respectively, a difference that was not significant. Based on this phase 3 study, 48 weeks of PEG-IFNα-2b and ribavirin combination therapy was approved for genotype 1 patients with a high viral load in Japan in 2004.

The adverse effects of ribavirin include hemolytic anemia and potential teratogenicity. Caution must be exercised with ribavirin administration when there is coexisting anemia or coronary heart disease. Contraception is also required during administration of ribavirin and up to 6 months after the end of its administration. Ribavirin is contraindicated for patients with renal failure, because it is excreted by the kidney and cannot be eliminated by dialysis.

Recent developments in PEG-IFN and ribavirin therapy

Coadministration of PEG-IFN and ribavirin has been established as the standard regimen of antiviral therapy for hepatitis C,[34] and the following questions next arise. How long should the dosing period be for this combination? How can its adverse effects be ameliorated and the treatment successfully completed? To what extent can this combination treatment be applied? Recent developments offer responses to these questions.

Exploring necessary and sufficient dosing periods: the impact of viral kinetics study

Usually the duration of coadministration of PEG-IFN and ribavirin is 48 weeks for difficult cases (e.g., genotype 1 patients with a high viral load) and 24 weeks for other cases, with expected SVR rates of approximately 50% and 80%, respectively.[32] Some studies have suggested that higher doses of ribavirin based on body weight are more effective for genotype 1, while a lower dose (fixed dose at 800 mg/day) is sufficient for viral genotypes other than genotype 1. To date, a variety of factors, both viral and host, that correlate with a sustained response to the combination therapy have been noted (Fig. 2). In contrast to viral factors, however, most host factors do not have a strong impact on the various treatment regimens. Recently, the viral kinetics after the start of therapy has been noted to be a useful early indicator of viral elimination, which is usually determined 24 weeks after the end of therapy.[20] To find out whether SVR is related to the rate of inhibition of viral replication after the start of PEG-IFN plus ribavirin combination therapy, Davis et al.[35] carried out a retrospective analysis of a controlled clinical study conducted by Manns et al.[26] In the clinical study, PEG-IFNα-2b (1.5 μg/kg per week) and ribavirin (800 mg/day) were coadministered to 511 patients with chronic hepatitis C for 48 weeks. If an early virologic response (EVR) is defined as a viral load decrease of 2 log or more or viral elimination after 12 weeks of treatment, then 71.8% of the patients who experienced EVR—74.4% of all patients—achieved SVR. Importantly, none of the patients who did not experience EVR achieved SVR. Similarly, with therapy with PEG-IFNα-2a (180 μg/week) plus ribavirin (1000 or 1200 mg/day, depending on body weight) for 48 weeks (n = 453), only 2 of 63 patients who did not experience EVR achieved SVR.[27] These findings show that EVR has negative predictive value, and therefore, if viral elimination is the aim of the treatment and if adverse effects cannot be negligible, the treatment should be discontinued in patients not displaying EVR. This "12-week rule" applies only to patients with viral genotype 1.[36]

The relationship between the time of becoming HCV-negative and SVR has also been examined in Japan in the above-mentioned clinical study[33] of PEG-IFNα-2b plus ribavirin. SVR rates for patients who became HCV-negative at 4, 12, or 24 weeks (23, 121, and 33 patients, respectively) were 100%, 71.1% and 36.4%, respectively. None of the 15 patients who experienced viral elimination after 24 weeks achieved SVR. Therefore, 24 weeks of additional administration to patients with no viral elimination within the initial 24 weeks produces no benefit.

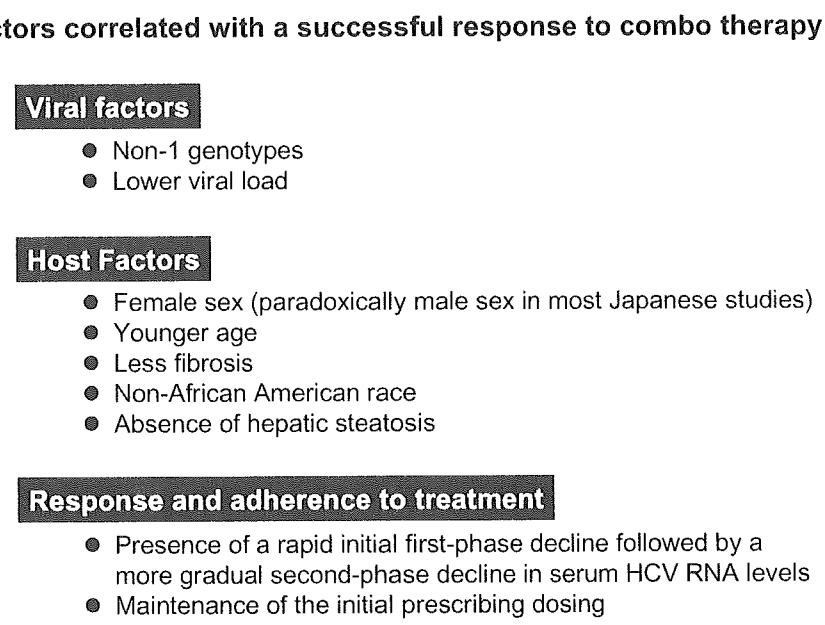

Fig. 2. Factors correlated with a successful response to combination therapy with pegylated interferon and ribavirin in chronic hepatitis C. HCV, hepatitis C virus

Genotype 1 patients who do not experience EVR are very intractable, as shown above. In other words, 48 weeks of therapy with PEG-IFN and ribavirin may be too short to maximize SVR in genotype 1 patients.[37] The usefulness of long-term administration for 48 weeks or longer is being investigated to improve the rate of achievement of SVR in such patients. Buti et al.[38] published a promising report on extending therapy with PEG-IFN plus ribavirin to 72 weeks for late virologic responders. They selected nine genotype 1 patients being treated with PEG-IFNα-2b (1.0μg/kg) plus ribavirin (800mg/day) who cleared HCV RNA between weeks 12 and 24 for therapy prolonged to 72 weeks. Eight patients completed therapy, and at week 24 of follow-up, seven maintained SVR and one had relapsed. A Spanish multicenter, randomized controlled study, in which patients with chronic hepatitis C who did not became HCV negative by 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (800mg/day) (about two-thirds of all patients) were randomized to groups receiving 48 weeks or 72 weeks of therapy, found that the group receiving 72 weeks of therapy achieved a significantly higher rate of SVR than the group receiving 48 weeks of therapy. On the other hand, a recent clinical trial showed that genotype 1 patients who were HCV RNA-negative after 4 weeks of coadministration of PEG-IFNα-2a (180μg/week) and ribavirin (1000 or 1200mg/day) achieved an SVR rate of 66% with a further 20 weeks of therapy.[39] Unfortunately, this study did not randomize the patients to compare 24 weeks of therapy with a 48-week therapy period. The study, however, does show that 24 weeks of therapy can achieve relatively high rates of viral elimination for these genotype 1 "super-responders."

For other, non-1 viral genotypes, studies are being done to identify a dosing period shorter than 24 weeks that can be used to achieve sufficient SVR. In one study, genotype 2 and 3 patients were given PEG-IFNα-2b (1.0μg/kg each week) and ribavirin (1000 or 1200mg/day, based on body weight), and those who experienced viral elimination after 4 weeks of therapy were assigned to 24-week or 12-week therapy groups. The results showed that the SVR rate for the 12-week group was the same as that for the 24-week group, indicating that 12 weeks of combination therapy is sufficient for these patients.[40] Similar data have also been reported for PEG-IFNα-2a (180μg/week) plus ribavirin (800 to 1200mg/day) therapy.[41]

As mentioned above, for treatment of non-1 viral genotypes and some genotype 1 patients, sufficient SVR rates can be achieved and unnecessary treatment avoided by adopting the dosing period by using the early viral inhibition effect as an indicator. The early viral kinetics can be also applied to identify more difficult to treat patients with viral genotype 1, who can then be given longer treatment to improve SVR rates (Fig. 3).

Reducing cytopenic effects and improving compliance: the use of hematopoietic growth factors

Patient compliance has been noted by many clinical studies to be the largest factor contributing to the therapeutic effect of PEG-IFN plus ribavirin combination

therapy (Fig. 2). Compliance can be divided into those factors related to patient adherence to the regimen and dose interruptions or modifications mandated by the physician in response to cytopenia, rash, gastrointestinal symptoms, or depression. McHutchison et al.[42] outlined an "80:80:80 rule" in genotype 1 patients; that is, the doses of PEG-IFN and ribavirin and the dosing period should exceed 80% of the initial plan to achieve a sufficient SVR rate. Early dose reduction within 12 weeks is more harmful than later dose reduction. To maximize viral clearance of the PEG-IFN and ribavirin combination therapy, countermeasures are needed against adverse effects to improve patient compliance.

Compared with IFN monotherapy, combination therapy is characterized by additional adverse effects represented by hemolytic anemia. If anemia occurs, the dose of ribavirin must be reduced or the administration of ribavirin must be discontinued. To help avoid this adverse effect, attention is being drawn to drug intervention with erythropoietin. An 8-week, double-blind study was conducted in which epoetin alpha 4000 U/week or a placebo was given to patients who experienced a decrease in hemoglobin (Hb) levels to 12 g/dl or less during coadministration of PEG-IFN and ribavirin in the United States, and the dose of ribavirin, Hb levels, and quality of life (QOL) were compared at the end of the study.[43] Compared with the placebo group, the reduction in Hb levels was significantly inhibited in the epoetin alpha group; thus, reduction of the ribavirin dose could be avoided. Inhibition of the reduction in Hb levels also improved QOL.[44] Similarly, granulocyte-colony stimulating factor (G-CSF) is expected to be useful for avoiding leukocytopenia induced by PEG-IFN and ribavirin combination therapy. Prevention of adverse effects with hematopoietic growth factors may be a promising measure to allow the maintenance of the therapy protocol and to improve therapeutic outcomes.

Challenging special patient groups: chronic hepatitis C with persistently normal ALT levels

Persistently normal ALT levels are observed in 20%–30% of chronic HCV-infected patients among the general public. Such patients are sometimes called asymptomatic HCV carriers. Most of them present a picture of histologically minimal or mild chronic hepatitis; it is rare for the liver to be normal. Progression of fibrosis is noted in fewer than 10% of the patients. For this reason, the expression "chronic hepatitis C patients with persistently normal ALT levels" is often preferred to "asymptomatic HCV carriers." There was strong resistance against using IFN therapy for such patients in the 1990s[45,46] for both active and passive reasons. The former included a lower viral elimination effect, or SVR, compared with general hepatitis C patients, and the report of abnormal ALT levels in a high percentage of patients due to IFN therapy in early studies of asymptomatic HCV carriers.[47,48] Recent studies have shown that IFN monotherapy[49] and IFN plus ribavirin combination therapy[50,51] can help patients with persistently normal ALT levels achieve the same level of SVR as patients with abnormal ALT levels. The percentage of patients who display an increase in the ALT level in response to IFN therapy is also lower than that in the early studies.[51] Therefore, the active reasons against using IFN therapy for patients with persistently normal ALT levels can no longer be supported. The passive reason, that there is no evidence of improved long-term prognosis in this patient group by IFN therapy, still remains.

HCV patients with normal ALT levels have been not eligible for large-scale clinical studies, causing there to be a deceptively low level of evidence regarding the efficacy of antiviral therapy in such patients. However, the potential importance of antiviral therapy for such patients has been gaining attention in recent years, and an international, multicenter, randomized, controlled study of PEF-IFNα-2a plus ribavirin combination therapy has been conducted.[52] Eligible participants were 491 HCV RNA-positive patients whose ALT levels measured three times or more at intervals of at least 4 weeks did not exceed the upper limit of the normal ALT range. The patients were randomized at the proportion of 3:3:1 into three groups: patients receiving 24 weeks of therapy with PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), those receiving 48 weeks of PEF-IFNα-2a (180 μg/week) and ribavirin (800 mg/day), and a control group that did not receive any treatment. Acute exacerbation of ALT levels that exceeded ten times the upper limit was observed in two patients (one in the 24-week therapy group and one in the control group). The results regarding treatment effectiveness were identical to those for chronic hepatitis C patients with high ALT levels previously published by Hadziyannis et al.[32] Thus, a dosing period based on the algorithm established for chronic hepatitis C patients with abnormal ALT levels can be recommended for PEG-IFN plus ribavirin combination therapy for HCV-infected patients with persistently normal ALT levels.

Such findings strongly suggest that HCV-infected persons with persistently normal ALT levels should be considered eligible for IFN therapy. The 2004 American Association for the Study of Liver Diseases (AASLD) best-practice guideline[36] recommended as follows: "Regardless of serum aminotransferase levels, the decision to initiate therapy with interferon and ribavirin should be individualized based on the severity of liver diseases by liver biopsy, the potential serious side effects, the likelihood of response, and the presence of comorbid