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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 (AdT β -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-, AdT β -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 AdT β -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 AdT β -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 lymphocytes³ 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 (AdT β -TR),¹⁰⁻¹² or bacterial β -galactosidase (AdLacZ)¹⁹ under a CA promoter comprising a cytomegalovirus enhancer and a chicken β -actin promoter²⁰ 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 20,000g 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 be-

tween 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, AdT β -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 μ g/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 \times) 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.

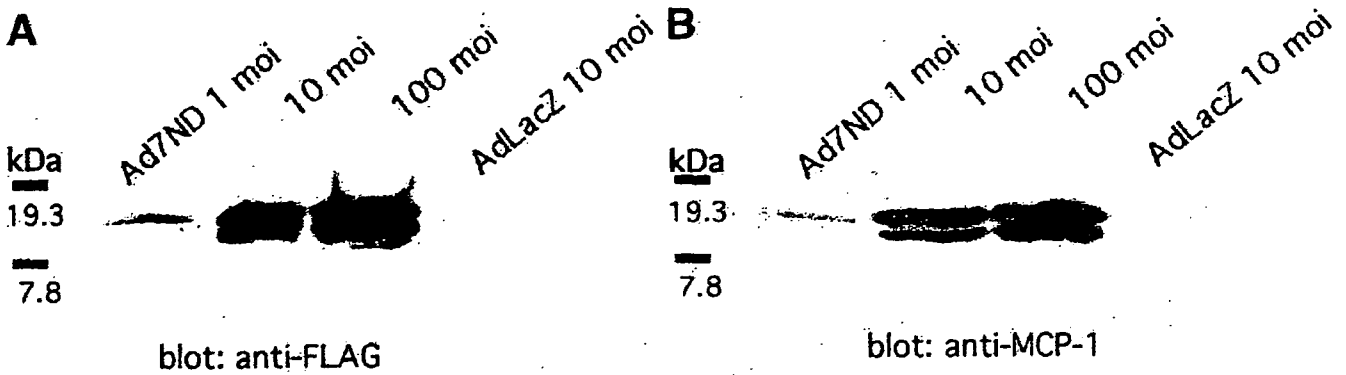


Figure 1. A mutated form of MCP-1 (7ND) is secreted from cells infected with Ad7ND. COS cells were infected either with Ad7ND or with AdLacZ at the indicated MOI. After 48 hours, the culture media were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and analyzed by Western 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

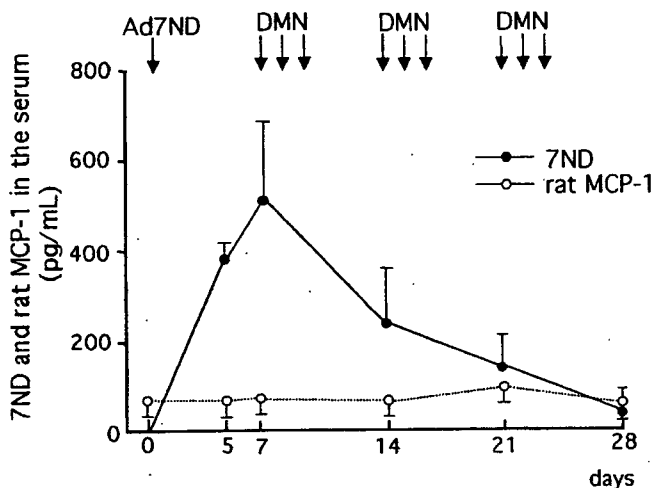
In the culture medium from Ad7ND-infected COS cells, 7ND was readily detectable in an MOI-dependent manner, as assessed by Western blotting analysis (Figure 1). Human 7ND and endogenous rat MCP-1 proteins were measured in sera (Figure 2A) and liver

extracts (Figure 2B) 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:

A (serum)



B (liver)

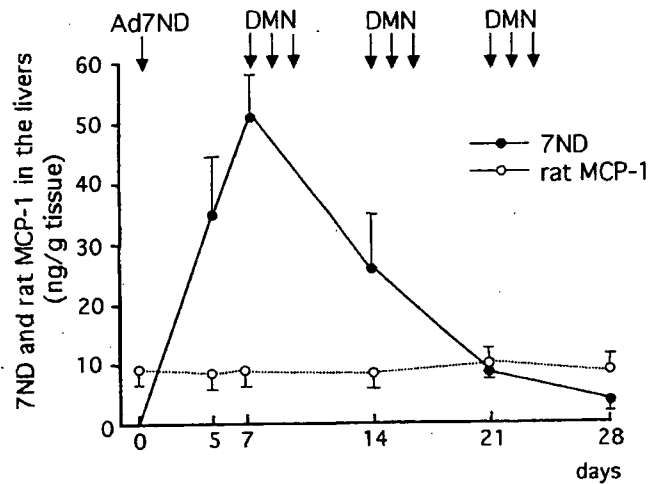


Figure 2. Amounts of human 7ND and rat MCP-1 in the sera (A) and livers (B) of DMN-injured rats. Rats were given a single infusion of Ad7ND (or saline infusion) via the tail vein. Seven days later, rats were subjected to a 3-week DMN treatment (shown as arrows). Rats were killed 5, 7 (just before the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

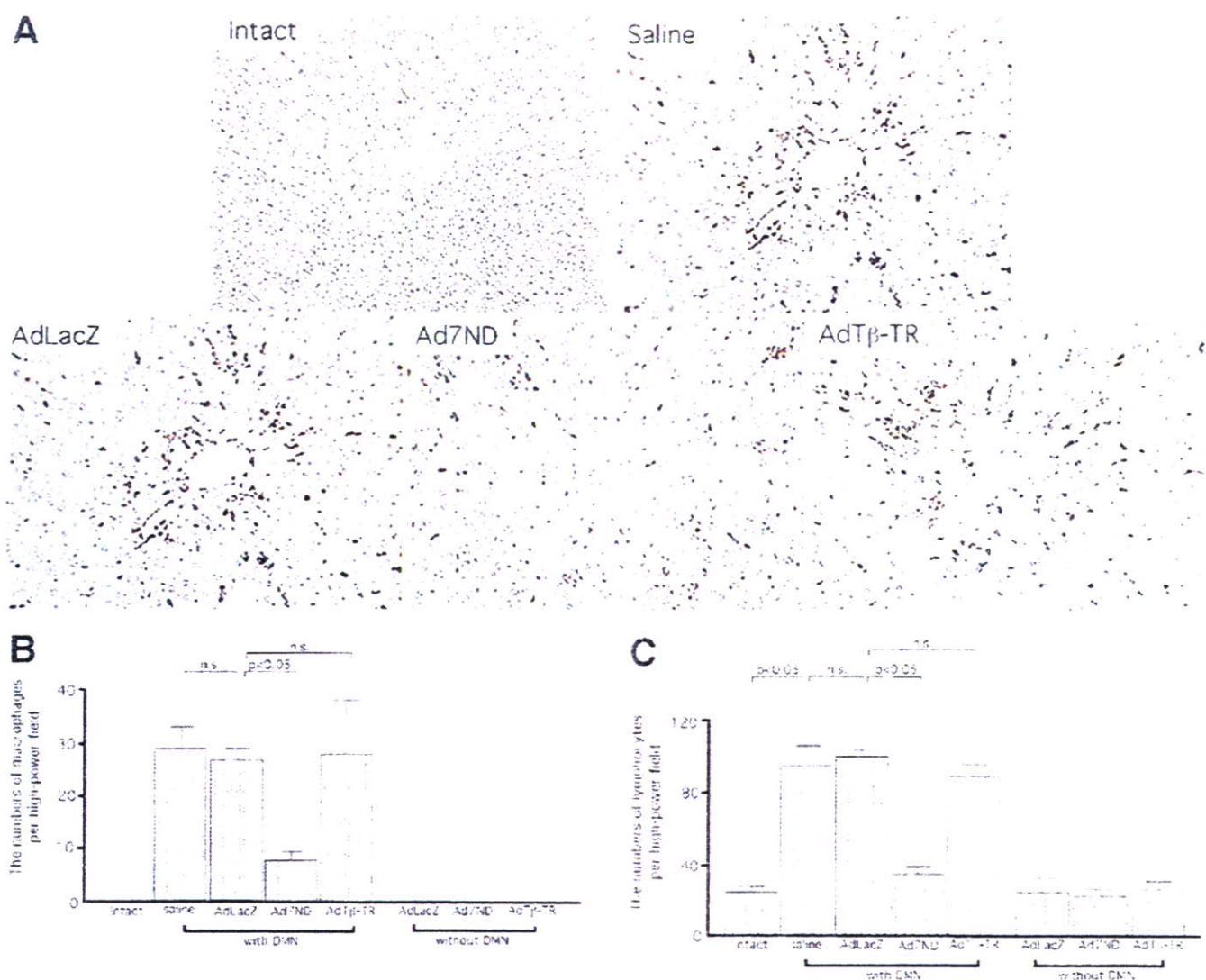


Figure 3. Infiltration of macrophages and lymphocytes into DMN-treated livers. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdT β -TR via the tail vein. Seven days later, some rats were administered DMN once (shown as *with DMN*) and some rats were not administered DMN (shown as *without 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 \times). 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 \pm 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 (AdT β -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, AdT β -TR-infected, or saline-injected rats; there were no differences among these 3 groups. However, macrophages were greatly reduced in Ad7ND-treated livers (Figure 3A).

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 AdT β -TR-treated livers, and only 7.1 ± 1.2 in Ad7ND-treated livers (Figure 3B). Similarly, the numbers of lymphocytes (histology not shown) were 98 ± 7.5 in saline-treated livers, 101 ± 2.5 in AdLacZ-treated livers, 98 ± 5.5 in AdT β -TR-treated livers, and only 40 ± 3.5 per high-power field in Ad7ND-treated livers (Figure 3C). Without DMN treatment, neither macrophages nor lymphocytes (histology not shown) were increased in the livers of AdLacZ-infected, AdT β -TR-infected, and

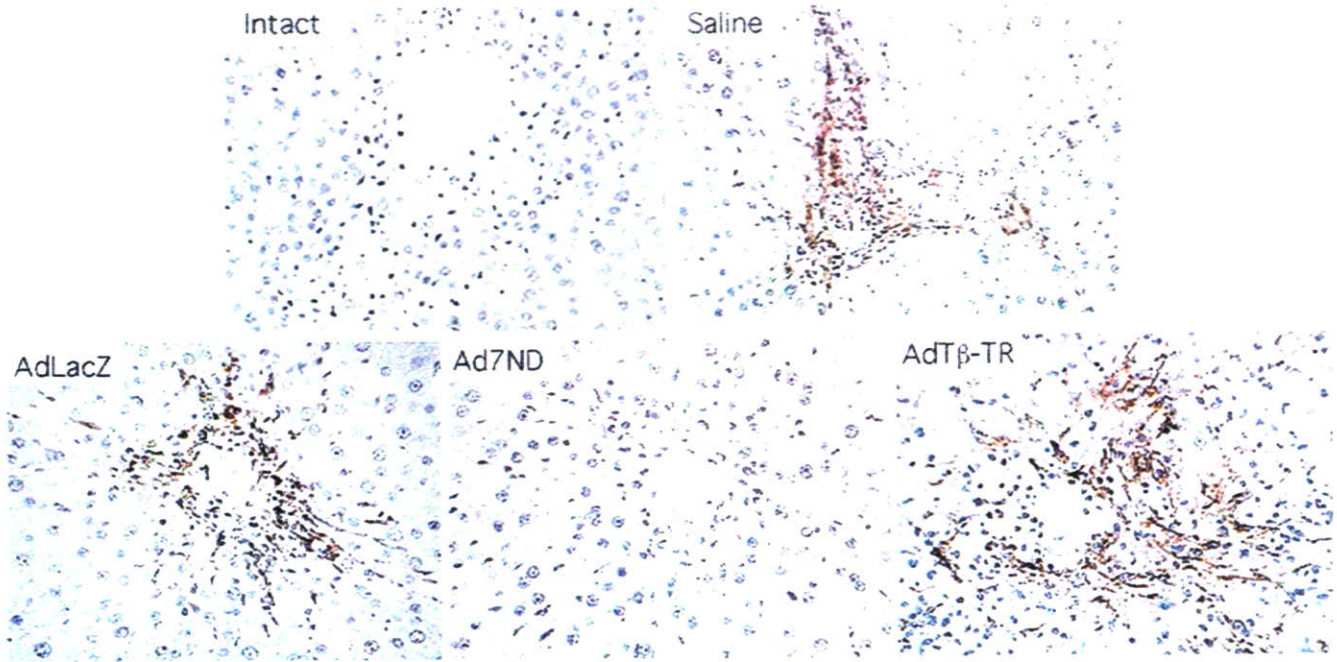


Figure 4. α -Actin-positive cells in DMN-treated livers. Rats were treated with either an adenovirus or saline as described in the legend to Figure 3 and then subjected to DMN for 3 consecutive days. One day after the last DMN injection (the fourth day), livers were examined by immunohistostaining against α -actin (original magnification, 200 \times). 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 3B 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 AdT β -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 AdT β -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 AdT β -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 AdT β -TR-treated livers showed a fibrotic area that was markedly smaller than that seen in

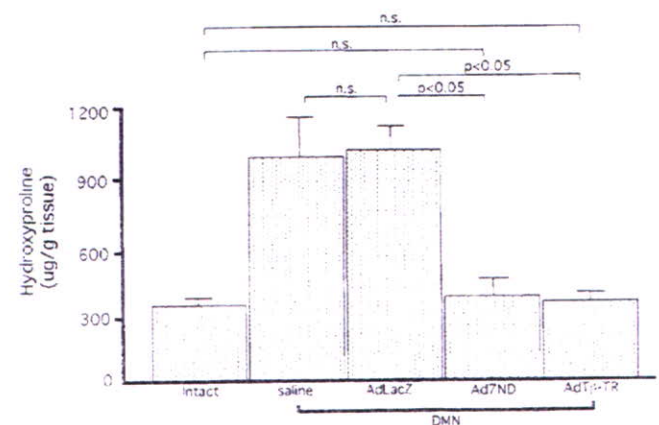


Figure 5. Hydroxyproline content of DMN-treated livers. Rats were treated with either adenovirus or saline as described in the legend to Figure 3 and then subjected to a 3-week DMN treatment. Hydroxyproline content of livers is shown as mean \pm SD. Three samples from each of 4 rats were analyzed for 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.

^aP < .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 7A). Immunohistostaining against α-actin confirmed that the TUNEL-positive cells in the AdTβ-TR-treated livers (Figure 7A) were indeed α-actin positive (Figure 7B).

Discussion

Inflammation induces infiltration by leukocytes and monocytes/macrophages into inflamed tissues.¹ Tis-

sue remodeling or fibrosis then follows the inflammation. MCP-1, one of the CC chemokines, attracts monocytes/macrophages bearing CCR2.¹¹ 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.^{13,14,15} In the Ad7ND-treated rats, DMN-induced infiltration by macrophages and lymphocytes into injured livers was markedly suppressed (Figure 5), 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 5 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

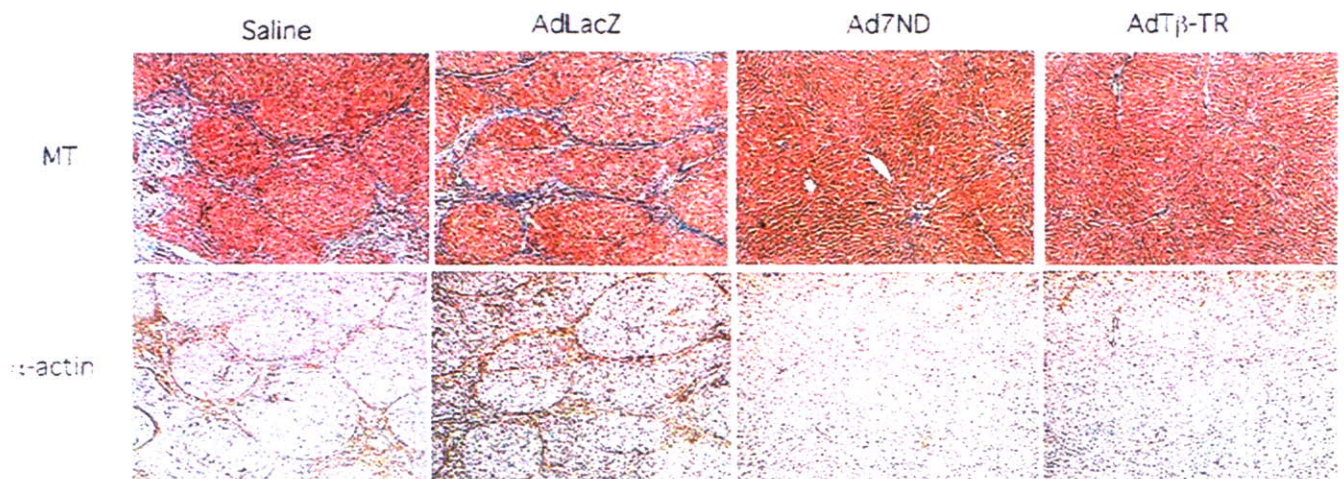


Figure 6. Histology of livers after a 3-week DMN treatment. Rats were treated as described in the legend to Figure 1. Liver sections were histologically examined with the aid of Masson trichrome staining (MT) or by immunohistostaining against α-actin (original magnification, 100×). Similar histology was seen in all 4 rats in each group.

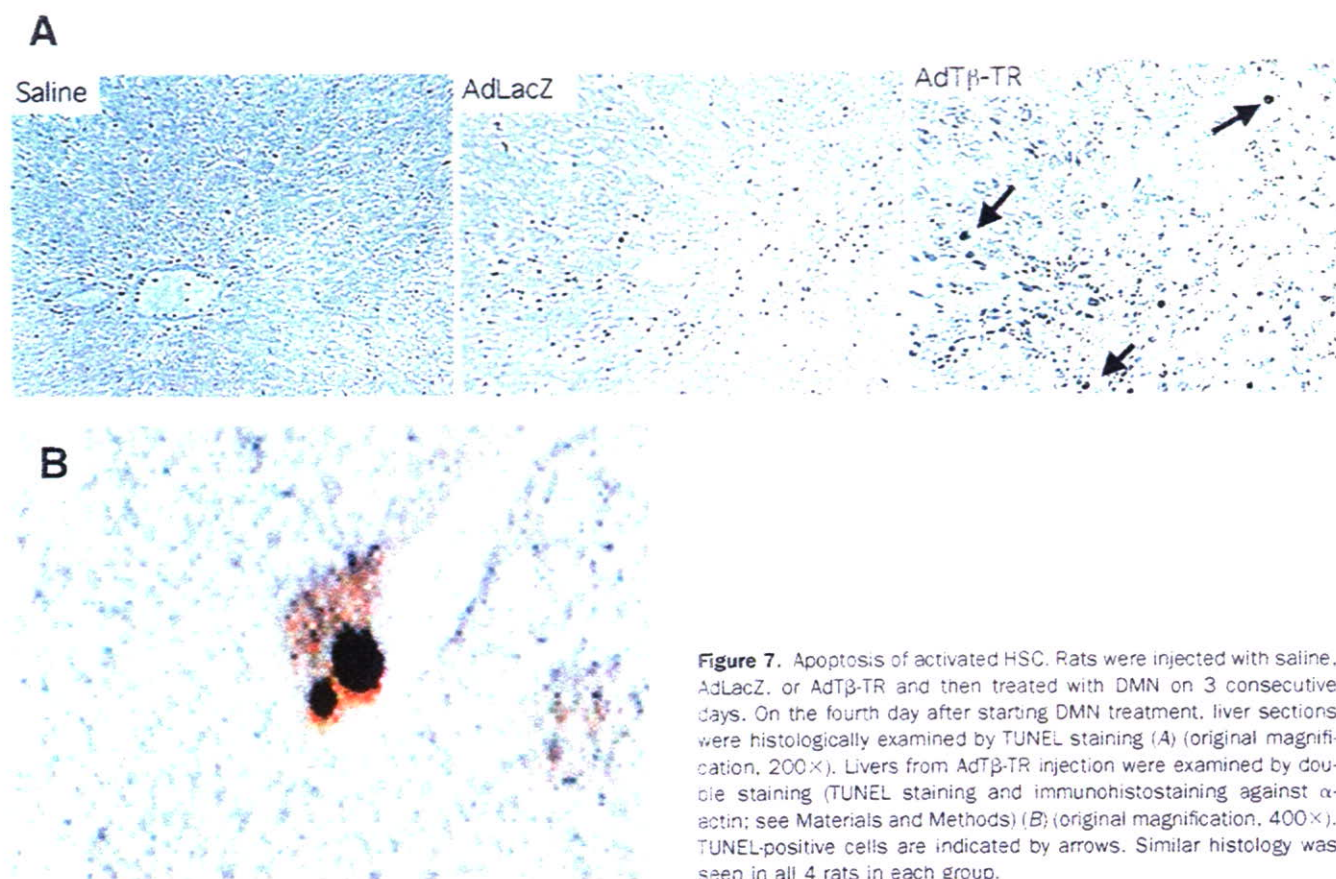


Figure 7. Apoptosis of activated HSC. Rats were injected with saline, AdLacZ, or AdT β -TR and then treated with DMN on 3 consecutive days. On the fourth day after starting DMN treatment, liver sections were histologically examined by TUNEL staining (A) (original magnification, 200 \times). Livers from AdT β -TR injection were examined by double staining (TUNEL staining and immunohistostaining against α -actin; see Materials and Methods) (B) (original magnification, 400 \times). 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^{11,12} 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).¹³ 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 AdT β -TR-treated livers (Figure 5); indeed, the numbers of activated HSC were the same among saline-treated, AdLacZ-treated, and AdT β -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,^{14,15} so the possibility can be excluded that all of these activated HSC were uninfected with AdT β -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 AdT β -TR-treated livers, but not in the AdLacZ- or saline-injected ones, activated HSC were in apoptosis (Figure 7). Saile et al.¹⁶ 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.¹⁷ 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 AdT β -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 al²⁸ 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/mL²⁵ (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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Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis C virus by PD98059

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Abstract

Translation initiation of hepatitis C virus (HCV) occurs in an internal ribosome entry site (IRES)-dependent manner. We found that HCV IRES-dependent protein synthesis is enhanced by PD98059, an inhibitor of the extracellular signal-regulated kinase (ERK) signaling pathway, while cellular cap-dependent translation was relatively unaffected by the compound. Treatment of cells with PD98059 allowed for robust HCV replication following cellular incubation with HCV-positive serum. Though the molecular mechanism underlying IRES enhancement remains elusive, PD98059 is a potent accelerator of HCV RNA replication.

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Keywords: HCV; IRES; ERK; Replication

Introduction

Hepatitis C virus (HCV), a member of the family Flaviviridae, is an enveloped virus with a positive-, single-stranded, 9.6-kb RNA genome (Murphy et al., 1995). The virus is the major causative agent of non-A, non-B hepatitis (Choo et al., 1989) and an estimated 170 million people throughout the world are persistently infected. Although acute phase HCV infection, in most cases, is asymptomatic, the virus frequently establishes a persistent infection, which is associated with serious clinical diseases such as chronic hepatitis followed by liver cirrhosis and hepatocellular carcinoma (Goodman and Ishak, 1995).

Like other positive-stranded RNA viruses, the 5'-untranslated region (UTR) of HCV RNA genome functions as an internal ribosomal entry site (IRES) and mediates translation initiation in a cap-independent manner (Tsukiyama-Kohara et al., 1992). Nearly the entire 5'-UTR (340 nt) and a short sequence of the coding region downstream of the initiator AUG codon of the HCV genome serve as an IRES (Honda

et al., 1996). Unlike encephalomyocarditis virus (EMCV) or poliovirus, the 5' end of the HCV genome is modified by neither cap structure nor VpG but bears a phosphate residue (Takahashi et al., 2005).

Molecular biological investigations of HCV have been hampered for a long time because of the lack of cell culture system that efficiently supports HCV replication. However, establishment of an HCV subgenomic replicon cell culture system in 1999 (Lohmann et al., 1999) allowed for such studies to be undertaken. The subgenomic replicon RNA is composed of, in this order, the HCV 5'-UTR containing an IRES, neomycin phosphate transferase or luciferase gene, HCV nonstructural (NS) proteins 3 through 5B directed by an EMCV IRES and the HCV 3'-UTR. As the replicon RNA replicates autonomously in cultured cells, the system provides a unique tool to analyze the molecular mechanisms governing viral genome replication and protein synthesis. Additionally, this system facilitates the screening of anti-HCV compounds.

PD98059 was identified as a potent inhibitor of mitogenic-extracellular signal-regulated kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway and has been widely used as a specific inhibitor of the pathway. The MEK-ERK pathway is elicited by broad

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range of growth factors or hormones and plays a crucial role in various events including cell growth promotion, differentiation, cell death and morphogenesis in eukaryotic cells (reviewed in Robinson and Cobb, 1997).

In this study, we demonstrate that PD98059 enhances HCV IRES-dependent translation. Because several lines of evidence suggest that IRES-mediated translation regulates replication in cultured cells (Lerat et al., 2000; He et al., 2003) and in vivo (Lott et al., 2001; Laporte et al., 2003; Forton et al., 2004), we examined the effect of PD98059 on viral replication. Although the RNA levels in replicon cells were relatively unaffected, PD98059 increased viral RNA levels in cultured cells infected with HCV-positive serum. Our results provide insight into the mechanisms of HCV IRES-dependent translation initiation and, in addition, suggest a simple infection system in cultured cells that supports HCV replication very efficiently.

Results

Enhancement of luciferase-replicon or HCV IRES by PD98059

We previously developed a highly efficient subgenomic HCV replicon system (Murata et al., 2005). Briefly, we used cured cells (curedMH14) as a host cell line, and the adaptive mutations were introduced into the subgenomic replicon construct for efficient replication. The luciferase gene was then placed under the control of the HCV IRES for rapid, quantitative and sensitive detection (Fig. 1, LMH14RNA). We have used this system to screen for compounds that inhibit HCV IRES-mediated translation. Treatment with IFN- α , IL-1 β , cyclosporin A (CsA) or TGF- β , all factors known to repress HCV replication (Blight et al., 2000; Zhu and Liu, 2003; Watashi et al., 2003; Murata et al., 2005), reduced the observed luciferase activity (Fig. 2A), demonstrating the effectiveness of this system. Conversely, the compound PD98059 increased the luciferase activity by 348% compared to vehicle (DMSO)-treated control (Fig. 2A). The increase in luciferase activity induced by PD98059 was not apparent at 6 h after compound addition, but the activity was significantly elevated by 12 h and remained high for at least 3 days (Fig. 2B). Since PD98059 is an inhibitor of the MEK-ERK pathway, we examined its effects on ERK phosphorylation (Fig. 2C). PD98059 treatment blocked ERK phosphorylation, but a clear band of phospho-ERK was seen in DMSO-treated cells due to growth factors present in the growth medium. Luciferase activity increased in a dose-dependent manner following PD98059 treatment (Fig. 2D). Treatment with the inhibitor at 30 μ M slowed cell growth (Fig. 6B) but did not put cells to death, while >30 μ M of the chemical resulted in a high degree of toxicity (not shown). We next wished to examine whether PD98059 specifically affected HCV IRES-mediated translation. Using a plasmid based di-cistronic vector

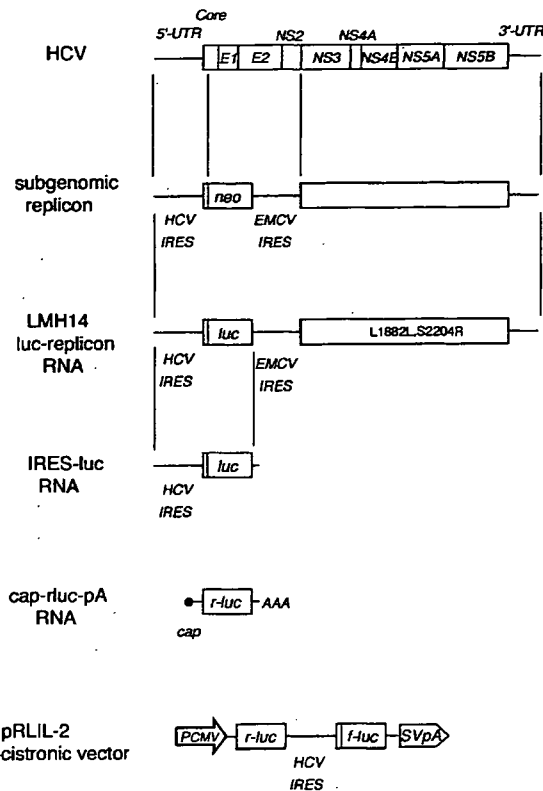


Fig. 1. Constructs used in this study. Schematic representation of the HCV RNA genome (HCV), G418-resistant subgenomic replicon (subgenomic replicon), subgenomic luciferase-replicon (LMH14), mono-cistronic luciferase expression vector with HCV IRES (IRES-luc), mono-cistronic renilla luciferase expression vector with cap and polyA (cap-luc-pA) or di-cistronic plasmid-based vector construct. The ORFs are depicted as open boxes.

(Fig. 1 pRLIL-2), we found that PD98059 increased the ratio of IRES-dependent translation to cap-dependent translation (Fig. 3B, 247 and 278% at 30 and 10 μ M, respectively). Translation downstream of a mono-cistronic mRNA was also enhanced, while cap-dependent translation was not affected (Figs. 3C,D). These results suggest that the positive response of the luciferase-replicon is primarily explained by increased IRES activity. Similar results were obtained when another inhibitor of the MEK-ERK signaling pathway, U0126, was used (Figs. 3E-H).

Effect of CGP57380 on HCV IRES

It has been known that either mitogen-activated protein kinase (MAPK)-interacting protein kinase (MNK) or eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) regulates translation initiation downstream of the MEK-ERK pathway (Raught and Gingras, 1999). We first examined the involvement of MNK in the IRES activation using CGP57380, a specific inhibitor of MNK (Knauf et al., 2001). ERK interacts with and phosphorylates MNK in

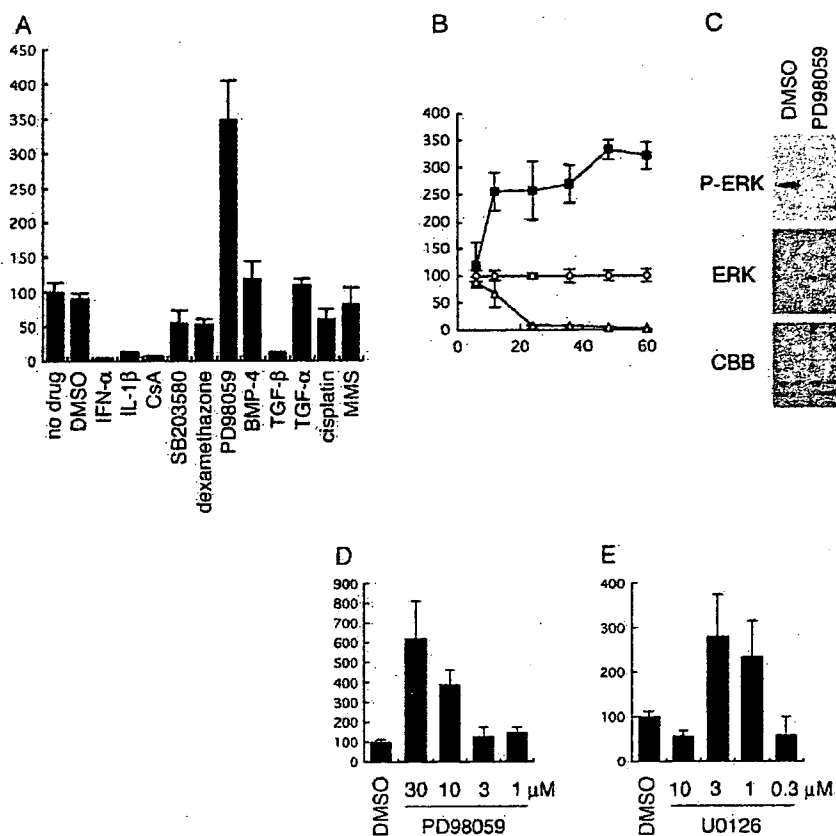


Fig. 2. Increased luciferase-replicon by PD98059 or U0126. (A) curedMH14 cells transfected with the luciferase-replicon RNA construct (LMH14) were treated with DMSO (0.1%), IFN- α (100 IU/ml), IL-1 β (10 ng/ml), cyclosporin A (CsA, 1 μ g/ml), SB203580 (10 μ M), dexamethazone (1 μ M), PD98059 (10 μ M), BMP-4 (10 ng/ml), TGF- β (2 ng/ml), TGF- α (1 ng/ml), cisplatin (1 μ g/ml) or MMS (methyl methanesulfonate; 0.1 mM). Three days later, cellular luciferase activity was measured. (B) curedMH14 cells transfected with the luciferase-replicon RNA construct were treated with DMSO (white circle), PD98059 (10 μ M, black box) or IFN- α (100 IU/ml). At the indicated times, cells were harvested for determination of luciferase activity. The activity of DMSO-treated cells was set to 100%. (C) Western blotting analysis of phospho-ERK (upper panel) and total ERK (middle panel) in cells treated with or without PD98059 (10 μ M) for 10 h. CBB staining pattern of the same blot is used as a loading control (lower panel). (D,E) Dose-dependence of MEK-ERK inhibitors on the activity of the luciferase-replicon. Cells transfected with the luciferase-replicon RNA construct were treated with varying concentrations of PD98059 (D) or U0126 (E), and luciferase activity was subsequently determined. The luciferase activity was shown with the SD value of three independent experiments.

response to growth or stress signals, respectively, and MNK phosphorylates eIF4E (Raught and Gingras, 1999).

Cell treatment with 20 μ M CGP57380 decreased the luciferase-replicon to 34% (Fig. 4A) and the ratio of IRES-dependent over cap-dependent value to 64% when dicistronic vector (Fig. 4B) was used. There was little to no effect of lower inhibitor concentrations on translation (Figs. 4A,B,C). In order to verify the effectiveness of CGP57380, we examined the activation of eIF4E by blotting with an anti-phospho-eIF4E antibody. Treatment with CGP57380 clearly eliminated eIF4E phosphorylation, and a partial reduction in eIF4E phosphorylation was seen following treatment with PD98059, even though total eIF4E levels were unchanged (Fig. 4D).

These data, combined with accumulating evidence (Scheper and Proud, 2002), suggest that eIF4E phosphorylation does not play a positive role in cap-dependent translation, and, moreover, it may limit cap-dependent

translation in cultured cells, although the physiological significance of eIF4E phosphorylation remains controversial. Nevertheless, drug-induced reduction in eIF4E phosphorylation did not enhance IRES-dependent translation compared to cap-dependent translation.

Effect of 4EBP on HCV IRES

An additional key translation regulator downstream of the MEK-ERK pathway is the eIF4E-binding protein 4EBP. When eIF4E is bound by 4EBP, ribosomes are not recruited to the cap structure and translation is blocked. Among the three isoforms, 4EBP1 is the best characterized. The binding of 4EBP1 to eIF4E is controlled by the phosphorylation state of 4EBP1, where the hypo/basal-phosphorylated form of 4EBP1 interacts tightly with eIF4E, but upon hyper-phosphorylation, 4EBP1 binding to eIF4E is inhibited (Gingras et al., 2001). mTOR has been reported to

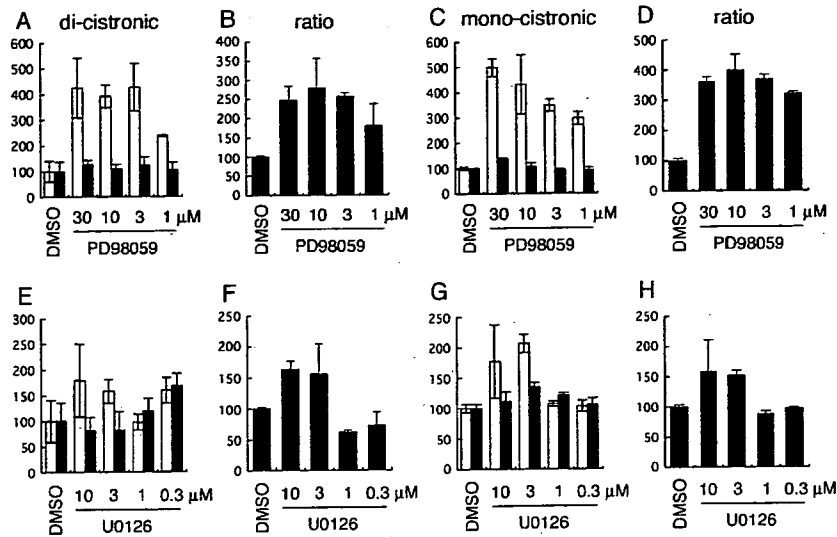


Fig. 3. Increased HCV IRES by PD98059 or U0126. Dose-dependence of MEK–ERK inhibitors on the activity of the di-cistronic pRLIL-2 vector (A,B,E,F) and mono-cistronic expression vectors (C,D,G,H). (A,E) Cells transfected with di-cistronic pRLIL-2 were incubated with varying concentrations of PD98059 (A) or U0126 (E), and luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (B,F) The results in panels (A) and (E) are shown as the ratio of IRES-dependent over cap-dependent value, respectively. (C,G) Cells transfected with mono-cistronic IRES-luc (white bar) and cap-rluc-pA (black bar) were incubated with varying concentrations of PD98059 (C) or U0126 (G), and luciferase activity was subsequently determined. (D,H) The results in panels (C) and (G) are shown as the ratio of IRES-dependent over cap-dependent value, respectively. The luciferase activity was shown with the SD value of three independent experiments.

phosphorylate 4EBP1 (Gingras et al., 1999), and, recently, Herbert et al. (2002) proposed that ERK is involved in the hyper-phosphorylation of 4EBP1.

We investigated a possible role for 4EBP1 in the observed IRES activation by PD98059. Exogenous expression of wild type or dominant active form of 4EBP1 (T46A, Mothe-Satney et al., 2000) elevated the luciferase-replicon to 420 and 325% of control levels, respectively, and PD98059 treatment enhanced these effects (Fig. 5A). A mutant form of 4EBP1 unable to interact with eIF4E (mBD, Mader et al., 1995), however, did not affect the luciferase-replicon activity (Fig. 4A). Luciferase expression driven by a di-cistronic vector resulted in a similar trend (Fig. 5C).

Both the wild type and mBD forms of 4EBP1 were hyper-phosphorylated (Fig. 5D). In the cell line used, Huh-7, endogenous 4EBP1 was not detected (Fig. 5D, vec). The expression levels of wild-type and T46A were reduced compared to mBD, likely as a result of the auto-suppression of cap-dependent translation by the wild type or T46A 4EBP1.

We next tried to eliminate endogenous 4EBP. Knock-down of 4EBP was confirmed following individual siRNA (Fig. 6A) or all siRNAs treatment (Fig. 6B). Among the different 4EBP isoforms, knock-down of 4EBP2 led to the strongest reduction in the luciferase-replicon (Figs. 6C,D) and the IRES/cap-translation in the di-cistronic vector (Figs.

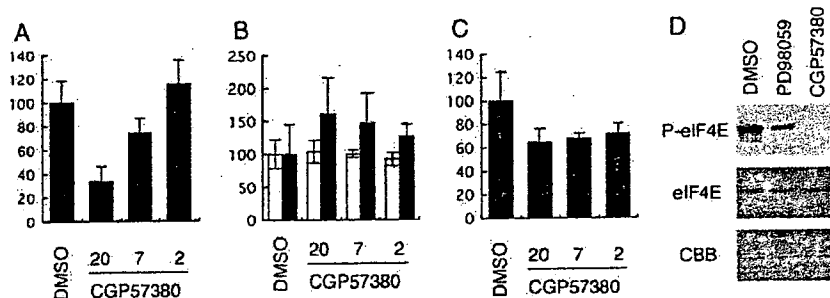


Fig. 4. Effect of CGP57380 on luciferase-replicon or HCV IRES. (A) Cells transfected with the luciferase-replicon RNA construct were treated with CGP57380 (μM). Three days after transfection, cells were harvested for determination of luciferase activity. The activity of DMSO-treated cells was set to 100%. (B) Cells transfected with di-cistronic pRLIL-2 were incubated with CGP57380, and luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (C) The results in panel (B) are shown as the ratio of IRES-dependent over cap-dependent value. The luciferase activity was shown with the SD value of three independent experiments. (D) Western blotting was performed to examine the phosphorylation of eIF4E (upper panel) and total eIF4E (middle panel) in cells treated with or without CGP57380 (20 μM). CBB staining pattern of the same blot is used as a loading control.

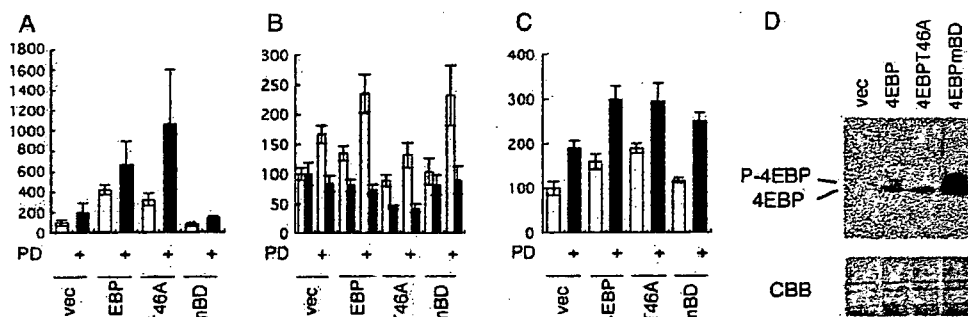


Fig. 5. Effect of exogenous expression of 4EBP on luciferase-replicon or HCV IRES. (A) Cells were transfected with the luciferase-replicon RNA construct together with empty vector (vec), vector for 4EBP1, 4EBP1T46A or 4EBP1mBD. 4EBP1T46A is a dominant-active form of 4EBP1, and 4EBP1mBD lacks the ability to bind with eIF4E. Cells were harvested for determination of luciferase activity after 3 days incubation with PD98059 (10 μ M). White and black bars indicate absence and presence of PD98059, respectively. The data are normalized by cotransfection with the pRL-TK. (B) Cells were cotransfected with dicistronic pRLIL-2 together with empty vector (vec), vector for 4EBP1, 4EBP1T46A or 4EBP1mBD. Luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (C) The results in panel (B) are shown as the ratio of IRES-dependent over cap-dependent value. White and black bars indicate absence and presence of PD98059, respectively. The luciferase activity was shown with the SD value of three independent experiments. (D) Western blotting was performed to examine the expression and phosphorylation state of 4EBP in cells using anti-4EBP antibody. CBB staining pattern of the same blot is used as a loading control.

6G,H). Huh-7 cells express higher levels of 4EBP2 compared to the other isoforms, and we hypothesize that this may account for the observed effect.

The above results suggest that 4EBP proteins, particularly 4EBP2 in this cell line, play an important role in HCV IRES-mediated translation. However, no evidence implicated 4EBP in the ERK-mediated modification of IRES activity because, even in the presence of the mBD mutant or the elimination of 4EBP isoforms, PD98059-mediated activation of IRES-dependent translation still occurred.

Effect of PD98059 on G418-resistant subgenomic replicon

Since IRES-mediated translation can regulate RNA replication in cultured HCV replicon cells (He et al., 2003), we tested the effect of PD98059 on G418-resistant replicon RNA replication. When monitored by either real-time RT-PCR (Fig. 7A) or Northern blotting (Fig. 7C), replicon RNA was increased up to 210% of vehicle-treated control by the administration with 30 μ M of PD98059 for 24 h. The replicon RNA levels decreased at 48 h or later probably because of the cell growth suppression (Fig. 7B). Additionally, PD98059 induced the production of viral protein NS5A (Fig. 7D). Although replicon RNA levels can fluctuate and are not the most stringent test, as Zhu and Liu (2003) also observed, the observed up-regulation of HCV replicon RNA and a viral protein at 24 h strongly suggests an effect of PD98059.

PD9805 promotes HCV multiplication in a model of HCV infection

To examine the effects of PD98059 on HCV replication, we infected curedMH14 (Fig. 8A), Huh-7 (B), OUMS-29 H-11 (C) or PH5CH8 (D) cells with HCV-positive serum for 1 day and incubated cells with either PD98059 or vehicle.

curedMH14 had been prepared by curing an HCV replicon cell line of replicon RNA (Murata et al., 2005). OUMS-29/H-11 is a human hepatocyte cell line, in which SV40 large T antigen and hepatocyte nuclear factor 4 (HNF4) had been introduced by stable transfection (Inoue et al., 2001), and PH5CH8 is a human hepatocyte line that had been immortalized with SV40 large T antigen (Ikeda et al., 1998).

HCV replication efficiency is highly dependent on the cell culture conditions, and poor infectivity can lead to little or no replication. However, HCV infectivity was dramatically improved by the addition of 30 μ M PD98059 (Fig. 8). With 30 μ M PD98059, virus RNA levels on day 5 were 162, 113 and 146% of the levels of day 1, whereas they were 0, 33 and 0% in curedMH14, OUMS-29 H-11 and PH5CH8 cells treated with DMSO, respectively. Thus, HCV replication was increased by 100-fold or more in curedMH14 and PH5CH8 cells on the fifth day. Huh-7 cells were not as permissive for viral infection under these conditions.

Discussion

In this study, we found that the addition of PD98059, an inhibitor of the MEK–ERK pathway, enhanced HCV IRES-dependent translation and HCV replication in cultured cells.

Multiple cellular factors bind directly to the HCV IRES including eIF3 (Sizova et al., 1998), the 40S ribosome (Otto et al., 2002), polypyrimidine tract-binding protein (PTB, Ali and Siddiqui, 1995), La autoantigen (Ali and Siddiqui, 1997) and heterogeneous nuclear ribonucleoprotein L (hnRNP L, Hahn et al., 1998). Some of these molecules may play a role in the PD98059-mediated activation of HCV IRES-dependent translation.

Several reports have suggested that translation driven by the HCV IRES (Honda et al., 2000), as well as other IRESes (Pyronnet et al., 2000; Cornelis et al., 2000), is highest in

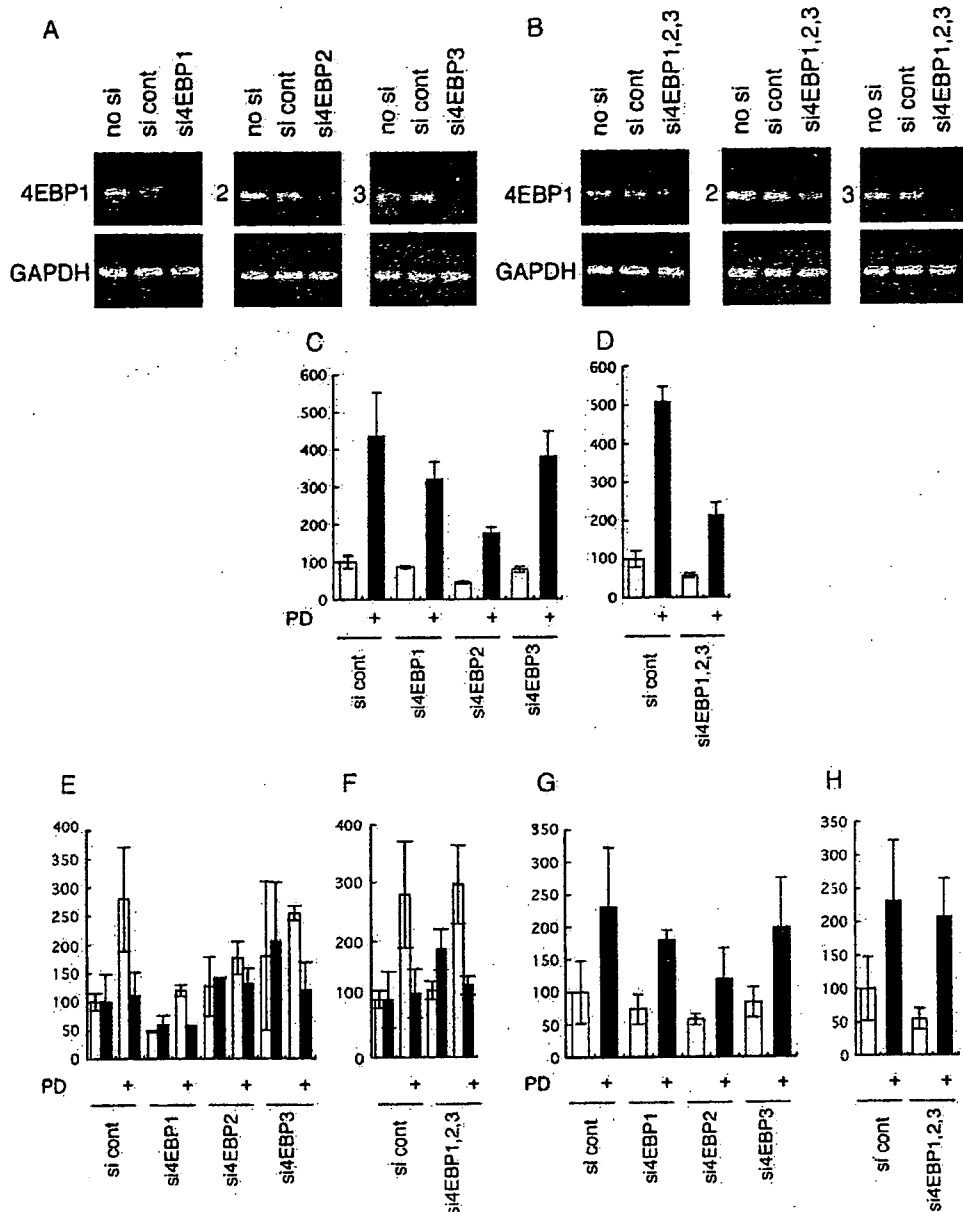


Fig. 6. Effect of RNAi knock-down of 4EBPs on luciferase-replicon or HCV IRES. (A) Cells were transfected with control siRNA (si cont) or si4EBP1, 2 or 3, independently. Total RNA was collected to examine the levels of relevant 4EBP RNA by RT-PCR. (B) Cells were transfected with control siRNA (si cont) or si4EBP1, 2 and 3, altogether. Total RNA was collected to examine the levels of every isoform of 4EBP RNA by RT-PCR. (C,D) Cells were cotransfected with LMH14 luciferase-replicon RNA construct and various siRNAs, independently (C) or together (D). Luciferase activity was determined after 3 days incubation with or without 10 μ M PD98059. White and black bars indicate absence and presence of PD98059, respectively. The data are normalized by cotransfection with the pRL-TK. (E,F) Cells were cotransfected with di-cistronic pRLIL-2 together with various siRNAs, independently (E) or together (F). Luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (G,H) The results in panels (E) and (F) are shown as the ratio of IRES-dependent over cap-dependent value. White and black bars indicate absence and presence of PD98059, respectively. The luciferase activity was shown with the SD value of three independent experiments.

the mitotic phase (G2/M) and relatively lower in other phases of the cell cycle. Since the MEK-ERK signaling pathway is largely suppressed in the G2 phase (Tamemoto et al., 1992), MEK-ERK signaling may also be a key regulator of this phenomenon.

In addition to ERK signaling, p38 MAPK and JNK signaling pathways are also involved in translation regulation. Cellular stress negatively affects cap-dependent protein synthesis (Patel et al., 2002), while EMCV (Hirasawa et al., 2003) or *c-myc* (Subkhankulova et al., 2001) IRES-

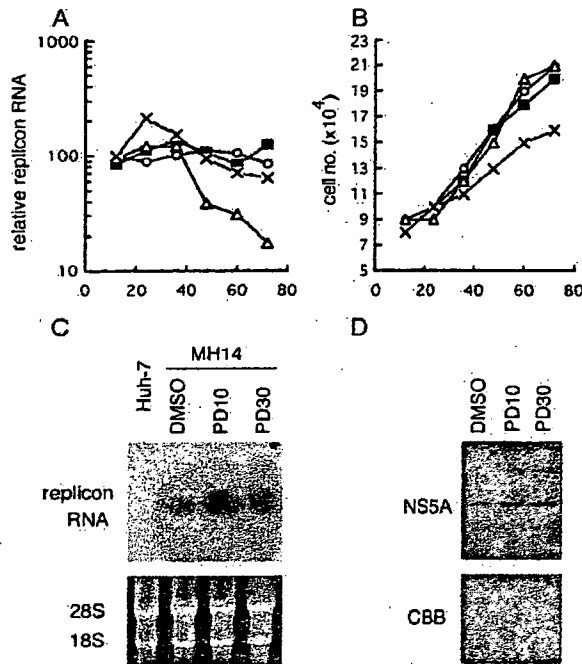


Fig. 7. Effect of PD98059 on G418-resistant subgenomic replicon. (A) MH14, a G418-resistant subgenomic replicon cell line, was treated with DMSO (white circle), 30 μ M PD98059 (X), 10 μ M PD98059 (black box) or 100 IU/ml IFN- α (white triangle) for 1, 3, 5 or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis. (B) In parallel with the experiments in Fig. 5A, cells were treated with DMSO (white circle), 30 μ M PD98059 (X), 10 μ M PD98059 (black box) or 100 IU/ml IFN- α (white triangle). Cell numbers were counted at the indicated time points. (C) Total RNA of cells treated for 1 day was also subjected to Northern blot analysis (upper panel). The ethidium bromide staining of ribosomal RNA is shown as an internal control (lower panel). (D) Total protein of cells treated for 1 day was harvested to examine the amount of NS5A (upper panel). CBB staining pattern of the same blot is shown as a loading control (lower panel).

dependent translation is elevated by these signals. Therefore, these signaling pathways may also affect HCV IRES-dependent translation.

A cell culture system supporting HCV replication has not existed for some time. When immortalized hepatocyte cell lines are infected with HCV, viral replication efficiency is not high despite high replication rates in patients. Many researchers have attempted to solve this problem. Ikeda et al. (1998) demonstrated that incubation of cells at lower temperature helps virus replication. Aizaki et al. (2003) used a three-dimensional hepatocyte culturing system. Others varied the bovine serum levels, vitamins, lipids or amino acid composition or the pH of the culture medium. We observed that freshly thawed cells with lower viability supported replication better than rapidly growing cells. We now propose a simple infection system that supports highly efficient HCV replication in cultured cells by adding PD98059 in the medium.

Cells isolated from human liver are cultured in conditions that substantially differ from the in vivo

environment and are often immortalized by oncogene expression. Consequently, many signaling pathways are likely aberrantly regulated in vitro. Among these pathways, it seems likely that ERK signaling is responsible for regulating HCV replication in cultured cells, and PD98059 may help mimic the in vivo environment and facilitate HCV replication by enhancing IRES-dependent translation.

Although treatment with PD98059 increased the replication of viral RNA in various cell lines when infected with HCV-positive serum (Fig. 8), replicon RNA levels were not increased under similar conditions (Fig. 7). The RNA copy number may explain these differences. PD98059 may not enhance the replication of replicon RNA because, in these systems, viral RNA and proteins are abundant even in the absence of the inhibitor. In cells infected with patient serum, highly efficient IRES-dependent translation may be essential for viral replication due to the low copy number of viral RNA per cell.

Mutations of serine residues within NS5A that affect the protein hyper-phosphorylation enhance replication of the virus replicon (Blight et al., 2000), and inhibitors of NS5A kinase(s) activate replication (Neddermann et al., 2004). Since the CMGC group of serine–threonine kinases has been implicated in the phosphorylation of NS5A (Reed et al., 1997), PD98059 might affect the

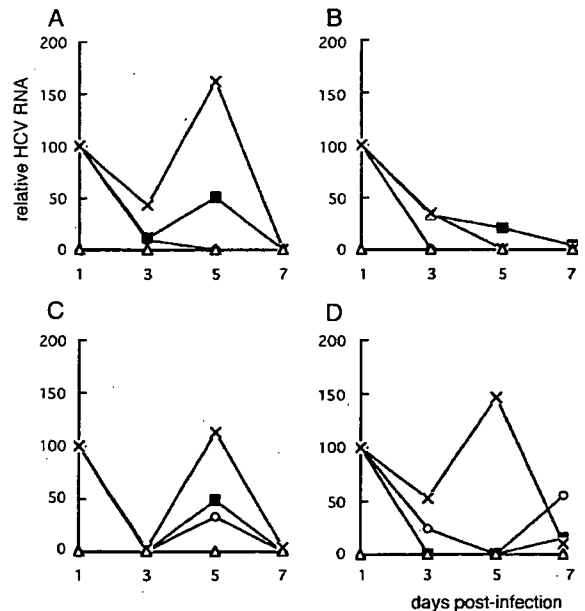


Fig. 8. Increased HCV multiplication by PD98059 in cells infected with HCV-positive serum. curedMH14 (A), Huh-7 (B), OUMS-29 H-11 (C) or PH5CH8 (D) cells were infected or mock-infected (white triangle) with HCV-positive serum for 1 day. After extensive washing with PBS, the cells were cultured with fresh medium supplemented with DMSO (white circle), 30 μ M PD98059 (X), 10 μ M PD98059 (black box). At the indicated times, total RNA was extracted, and the quantity of HCV RNA was determined by real-time RT-PCR analysis.

phosphorylation of the NS5A protein and thereby elevate replication. When we treated cells with PD98059, however, the levels of hyper-phosphorylated NS5A were not affected (not shown). This suggests that PD98059 activates viral replication through the enhancement of IRES-mediated translation but not through a reduction in phosphorylation state of NS5A.

Multiplication of influenza virus (Pleschka et al., 2001), borna disease virus (Planz et al., 2001), coxsackievirus (Luo et al., 2002), visna virus (Barber et al., 2002), human immunodeficiency virus (Montes et al., 2000), vaccinia virus (de Magalhaes et al., 2001), Epstein–Barr virus (Gao et al., 2001), cytomegalovirus (Rodems and Spector, 1998) and human herpesvirus-8 (Akula et al., 2004) are promoted by MEK–ERK signaling pathway activation. Activation of this pathway results in efficient cell cycle promotion, high cellular or viral gene production and increased availability of biomaterials, such as nucleotides or amino acids. Many of these viruses, therefore, likely exploit the cellular environment created through the activation of the MEK–ERK pathway. Interestingly, replication of the hepatitis B virus (HBV) is negatively regulated by the MAPK signaling pathway (Zheng et al., 2003). Because both HBV and HCV infect the same target organ, it is possible that both viruses have evolved similar means to exploit host signaling pathways. Much research is needed to identify the factors conferring organ specificity to HCV, however.

Materials and methods

Cell culture, antibodies and reagents

Huh-7 or curedMH14 cells (Murata et al., 2005) were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen, Carlsbad, CA) and 100 µg/ml penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA). MH14 replicon cells (Miyazari et al., 2003) were cultured in the same medium with 300 µg/ml G418 (Geneticin, Invitrogen, Carlsbad, CA). OUMS-29/H-11 cells (Inoue et al., 2001, Fukaya et al., 2001) were maintained in ASF-104 medium (Ajinomoto, Tokyo, Japan) with 100 µg/ml penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA), and PH5CH8 cells were cultured as described (Ikeda et al., 1998).

Rabbit anti-ERK, rabbit anti-phospho-ERK, rabbit anti-eIF4E and mouse anti-phospho-eIF4E antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-4EBP antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish-peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences (Piscataway, NJ). PD98059 and other inhibitors were obtained commercially from Calbiochem-Novabiochem (San Diego, CA).

Plasmid construction

The pLMH14, used to synthesize the luciferase-replicon LMH14 RNA and mono-cistronic IRES-luc RNA, has been described previously (Murata et al., 2005). The di-cistronic plasmid vector, pRLIL-2, was based on the pRL-CMV Vector (Promega, Madison, WI) and contains HCV IRES sequence (complete 5'-UTR sequence and initial part of the Core gene) plus the firefly luciferase sequence obtained from pGL2 Vector (Promega, Madison, WI).

The human 4EBP1 gene was cloned by RT-PCR into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) to obtain pcDNA4EBP. Primers used to clone the gene were 5'-cggaattcgatgtccggggcagcagctgc-3' and 5'-ctgactcgagttaaatgtccatctcaactgtg-3'. To generate pcDNS4EBPT46A and pcDNA4EBPmBD plasmids, mutations were inserted into pcDNA4EBP by PCR-based site-directed mutagenesis using the primers 5'-ctgggtacc-tcccggggcgggtgctgaagagcgtg-3' for T46A and 5'-gaggtacc-caggatcatctatgaccggaaattcgcggcggagtgctggaactc-3' for mBD. Bold letters in the primers denote the substituted nucleotides.

RNA synthesis *in vitro*

In order to synthesize the LMH14 luciferase-replicon RNA or mono-cistronic IRES-luc RNA, pLMH14 was digested with *Xba*I or *Kpn*I, respectively, and subjected to *in vitro* transcription using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions. Following DNase treatment, RNA was purified by lithium chloride precipitation. For production of mono-cistronic cap-rLuc-pA RNA, the pRL-TK Vector (Promega, Madison, WI) was cut with *Xba*I and transcribed *in vitro* using mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX) for capping. Poly(A) Tailing Kit (Ambion, Austin, TX) was then used for polyadenylation of the RNA.

Luciferase assay

Lipofection with RNA was performed using DMRIE-C reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA, including pRLIL-2, was transfected into cells using FuGENE6 reagent (Roche, Indianapolis, IN). Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Assays were performed in triplicate; standard deviations are denoted by bars in the figures.

Real-time RT-PCR analysis

Total RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The 5'-UTR of HCV genomic RNA was quantified with the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City,

CA) as described (Watashi et al., 2003) using the 5'-CGGGAGAGCCATAGTGG-3' (forward) and 5'-AGTAC-CACAAGGCCTTTCG-3' (reverse) primers and the fluorogenic probe 5'-CTGCGGAACCGGTGAGTACAC-3'. As an internal control, ribosomal RNA was quantified using TaqMan ribosomal RNA control reagents (Applied Biosystems, Foster City, CA).

Northern and Western blot analysis

Total RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan). Northern or Western blot analysis was performed as described previously (Kishine et al., 2002). The 1.5-kb *EcoRI* fragment of pNRRZ2 was used as the probe, which corresponds to the C-terminal half of the NS5A gene and N-terminal half of the NS5B gene.

In vitro HCV infection

The in vitro HCV infection experiment was carried out as described previously (Watashi et al., 2003). In short, cells were infected with the serum which was prepared from an HCV-positive blood donor. At 24 h post-inoculation, the cells were washed three times with PBS and maintained with fresh medium with DMSO or PD98059 until the extraction of the RNA sample.

siRNA

Sequences of siRNAs (Invitrogen, Carlsbad, CA) were as follows: 5'-aactcacctgtgaccaaaca-3' for 4EBP1, 5'-aagactccaaagtagaagtaa-3' for 4EBP2 and 5'-aagctggagtg-caagaactca-3' for 4EBP3. Before using, the siRNAs were dissolved in RNase-free water, denatured once at 98 °C for 1 min and annealed at 37 °C for 1 h. For electroporation of siRNA, 4×10^5 cells and 0.8 µg siRNA were suspended in 400 µl of OPTI-MEM (Invitrogen, Carlsbad, CA) and pulsed at 250 V and 950 µF using GenePulser (Bio Rad, Hercules, CA) at 4 °C. To evaluate the silencing effects of siRNAs, RT-PCR was performed using One-Step RT-PCR Kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's instruction. Primer sequences used were as follows: 4EBP1, 5'-cggaattcagatgctcggggcagcagctgc-3' and 5'-ctgactcaggt-taaatgtccatctcaaaactgtg-3', 4EBP2, 5'-cggaattcagatgctcgt-cagccggcag-3' and 5'-ctgactcaggtcagatgtccatctcgaac-3', 4EBP3, 5'-cggaattcagatgctcagctcactagctg-3' and 5'-ctgactc-gagttagatgtccattcaaatg-3', GAPDH, 5'-ttctcagatggg-gaaggtgaaggtcg-3' and 5'-ccggaattctggaggatctcgtcctg-3'.

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