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.

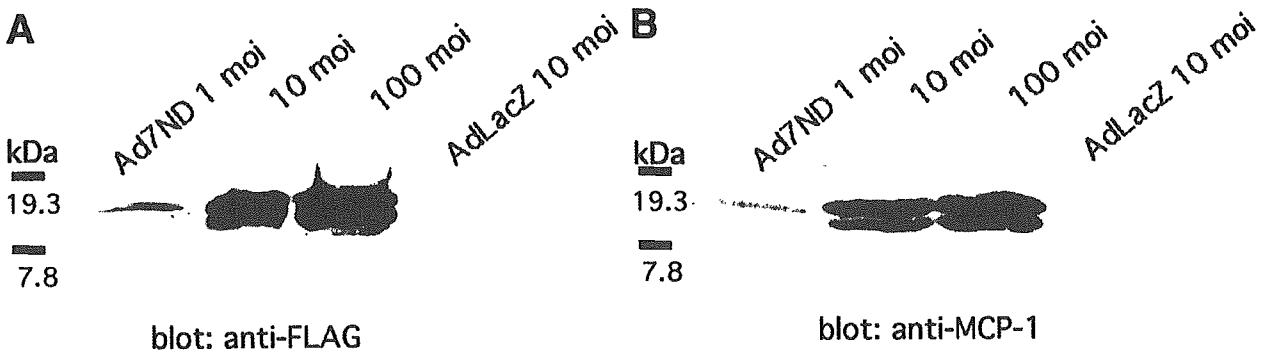

Figure 1. A mutated form of MCP-1 (7ND) is secreted from cells infected with Ad7ND. COS cells were infected either with Ad7ND or with AdLacZ at the indicated MOI. After 48 hours, the culture media were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and analyzed by Western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

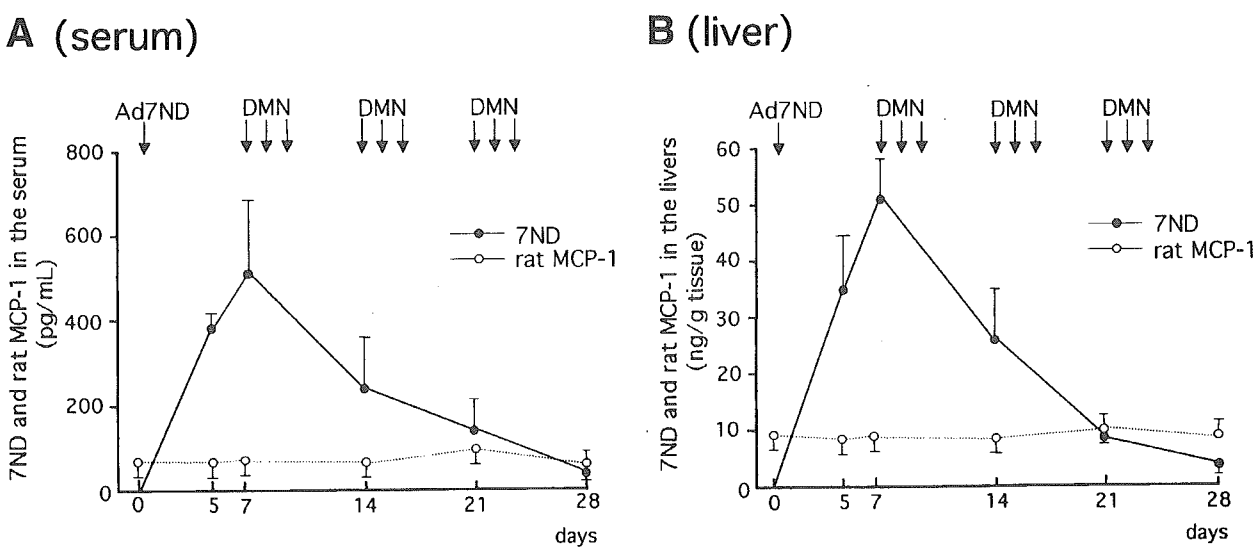

Figure 2. Amounts of human 7ND and rat MCP-1 in the sera (*A*) and livers (*B*) of DMN-injured rats. Rats were given a single infusion of Ad7ND (or saline infusion) via the tail vein. Seven days later, rats were subjected to a 3-week DMN treatment (shown as arrows). Rats were killed 5, 7 (just before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

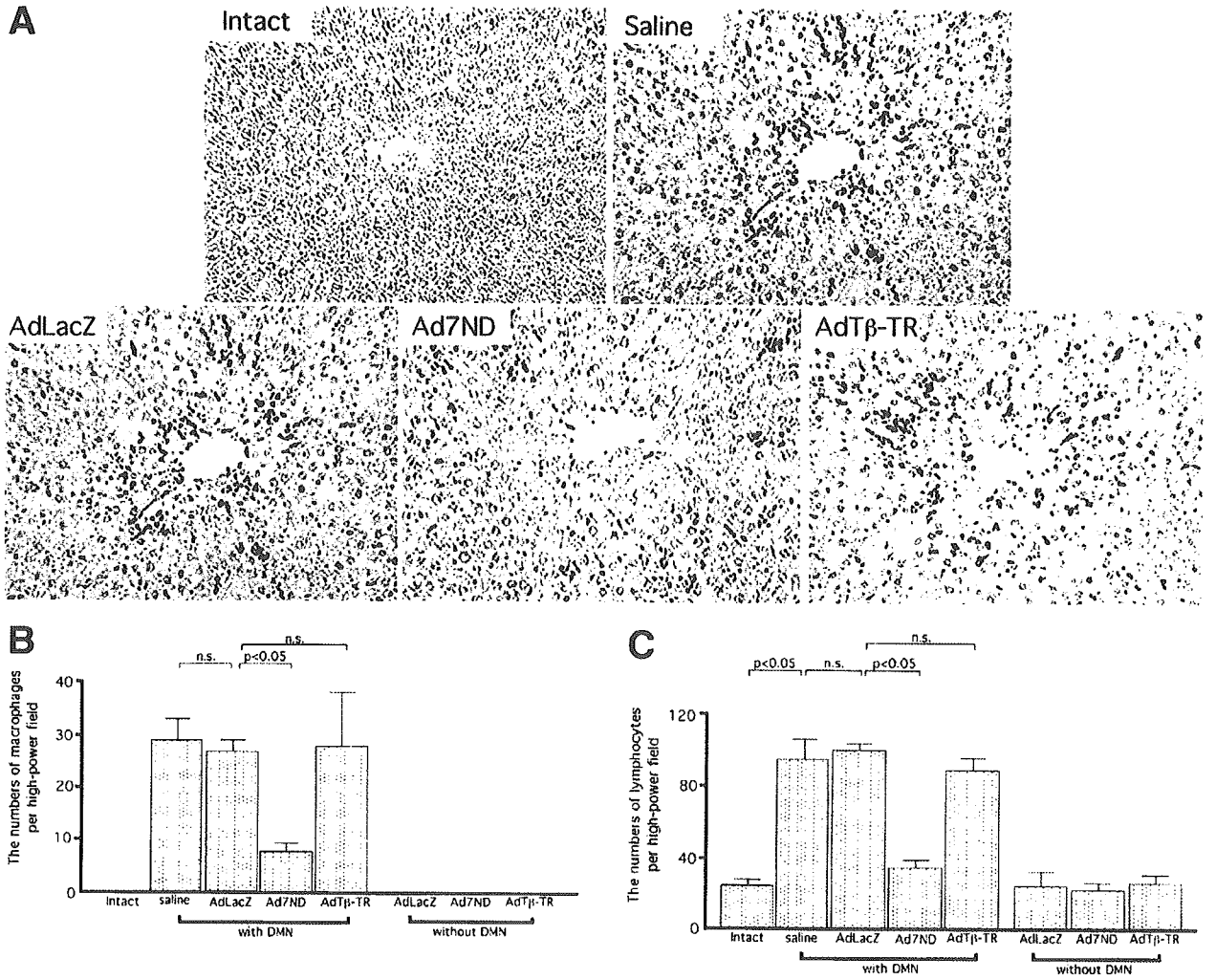

Figure 3. Infiltration of macrophages and lymphocytes into DMN-treated livers. Rats were given a single infusion of saline, AdLacZ, Ad7ND, or AdTβ-TR via the tail vein. Seven days later, some rats were administered DMN once (shown as *with DMN*) and some rats were not administered DMN (shown as *without 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

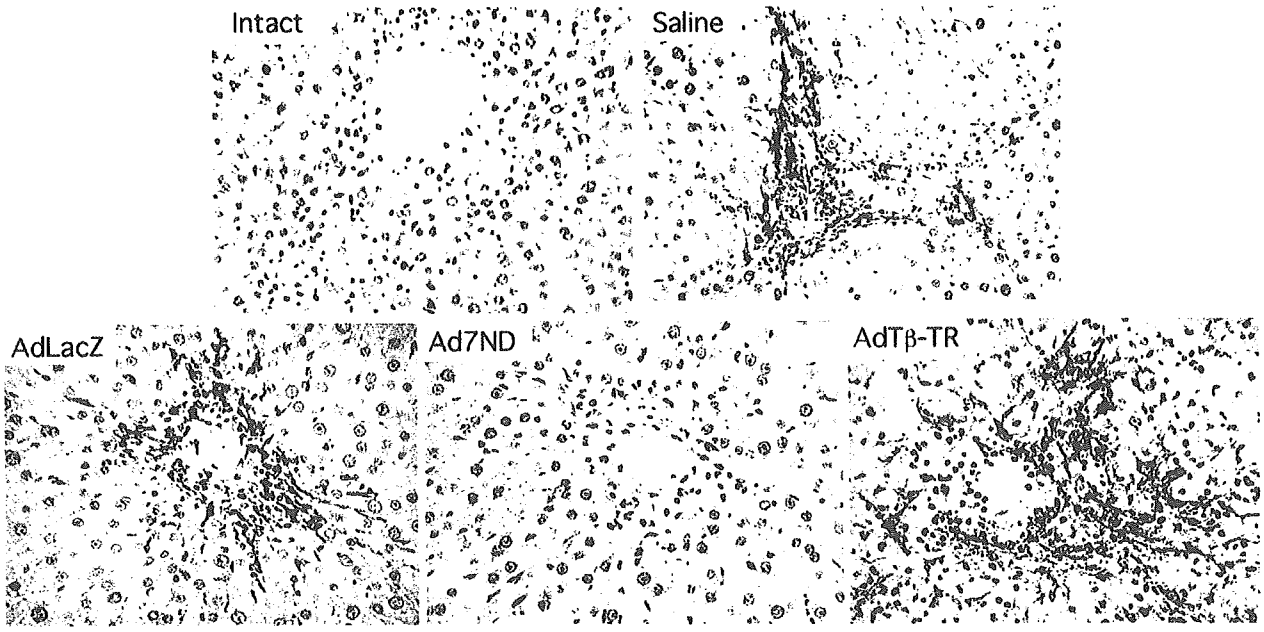

Figure 4. α-Actin–positive cells in DMN-treated livers. Rats were treated with either an adenovirus or saline as described in the legend to Figure 3 and then subjected to DMN for 3 consecutive days. One day after the last DMN injection (the fourth day), livers were examined by immunohistostaining against 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

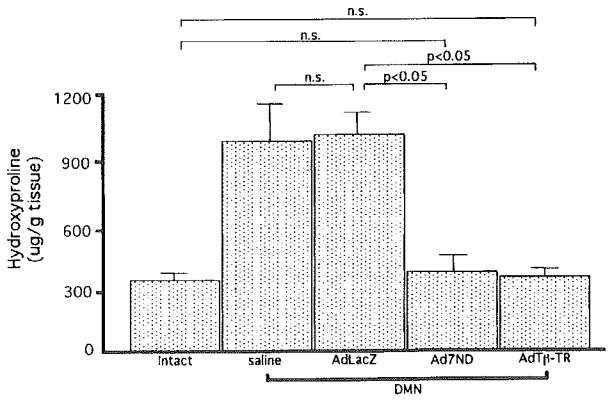

Figure 5. Hydroxyproline content of DMN-treated livers. Rats were treated with either adenovirus or saline as described in the legend to Figure 3 and then subjected to a 3-week DMN treatment. Hydroxyproline content of livers is shown as mean ± SD. Three samples from each of 4 rats were analyzed for each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

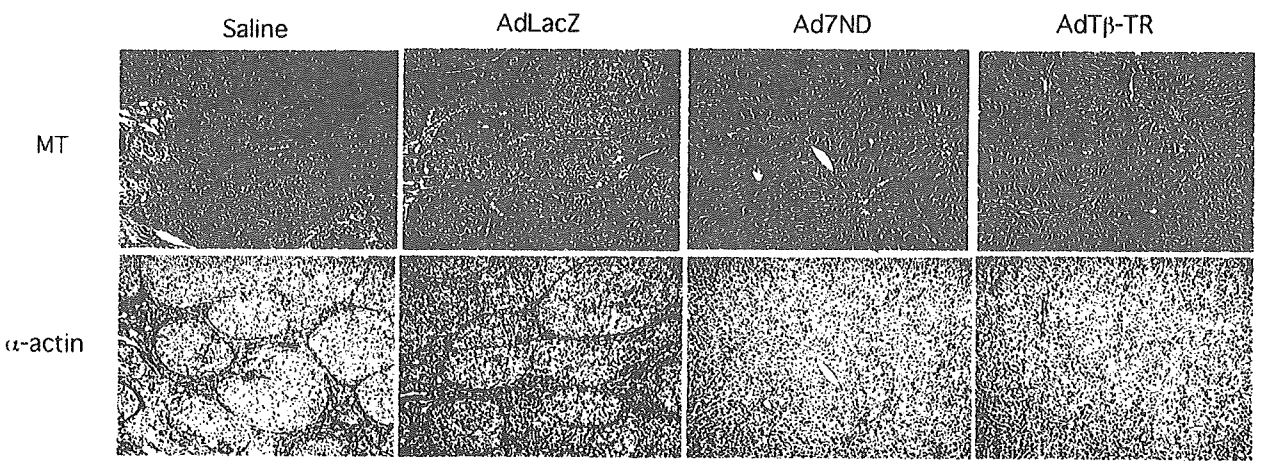

Figure 6. Histology of livers after a 3-week DMN treatment. Rats were treated as described in the legend to Figure 5. Liver sections were histologically examined with the aid of Masson trichrome staining (MT) or by immunohistostaining against α-actin (original magnification, 100×). Similar histology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

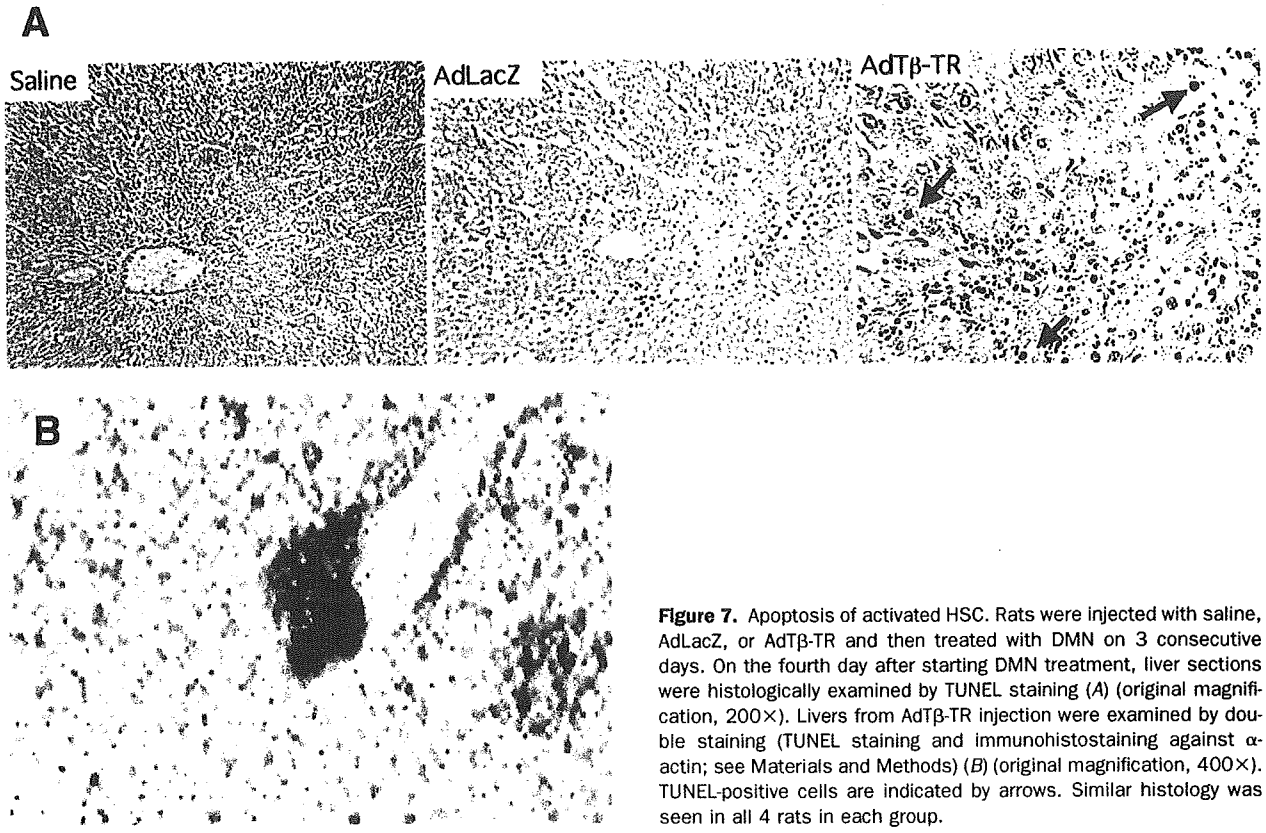

Figure 7. Apoptosis of activated HSC. Rats were injected with saline, AdLacZ, or AdTβ-TR and then treated with DMN on 3 consecutive days. On the fourth day after starting DMN treatment, liver sections were histologically examined by TUNEL staining (*A*) (original magnification, 200×). Livers from AdTβ-TR 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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EDITORIAL

Integration of hepatitis B virus DNA and hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common malignant human tumors worldwide.[1] Hepatitis B virus (HBV) and the hepatitis C virus (HCV) infections are etiologically linked to this cancer. HCC often emerges after the development of cirrhosis in patients with chronic HCV infection. In contrast, in patients with the HBV infection, HCC sometimes develop in non-cirrhotic patients, although the incidence is much lower than in patients with cirrhosis.[2] The HBV is a DNA virus that integrates into the chromosome of the host. In contrast, the HCV is a RNA virus that does not integrate into the host genome. It seems that the integration of the HBV into host chromosome(s) plays an important role in the development of HCC, especially in non-cirrhotic patients with HBV infection.

There are two major possible carcinogenic effects of HBV on the development of HCC. One is the direct effect of viral proteins such as HBx. In fact, this protein has been proven to be a transactivator that activates a variety of viral and cellular promoters. It has also been reported that HBx binds to p53 and inhibits p53-mediated cellular processes, including DNA binding, transcriptional transactivation and apoptosis. The viral proteins that might be produced as a result of integration and truncation of preS2/S and hepatitis B spliced proteins have also been reported to function as transcriptional modulators.[3] These proteins have been shown to modulate the transcriptional activation of cellular growth-regulating genes, to modify apoptosis and to inhibit nucleotide excision and the repair of damaged cellular DNA. The other possible carcinogenic effect of HBV is the integration of HBV-DNA into a host chromosome. This might interrupt cellular tumor suppressor genes, or cause alterations in the expression of cellular growth factors and/or apoptosis regulating factors. Although frequent activation of the N-myc oncogene has been reported in woodchuck hepatitis virus integration,[4] no apparent favored locus of human oncogene/tumor suppressor gene has been found. However, there are many reports that describe the alteration of cellular proliferation pathways (important in the control of cell signaling, proliferation and viability) as a result of the insertion of HBV-DNA. Recent reports have shown that in at least some hepatocellular carcinomas, the human telomerase reverse transcriptase (hTERT) gene is a non-random integration site of the HBV genome, which activates the hTERT transcription *in cis*.[5]

In this issue of the Journal, Huang *et al.*[6] reported a high incidence of HBV-DNA integration in tumor and non-tumor tissues of children with chronic HBV infection. They detected a higher incidence of the HBV integration using a sensitive inverse polymerase chain reaction (IPCR). This method was developed to amplify unknown sequences that flank a region of a known sequence (Fig. 1)[7,8]. This technique is very sensitive because a nested polymerase chain reaction is used for amplification of DNA. However, in some instances, it fails to detect integrated HBV-DNA. For example, integrants that do not contain a full four site of primer escape detection. Alternatively, the flanking region that does not contain an employed restriction enzyme digestion site near the integration portion fails to be amplified. The latter possibility is less likely because the employed restriction enzymes are four base recognizing cutters, the restriction site often appearing in common nucleotide sequences. However, it sometimes the restriction enzyme site is too near to the integration site and consequently, the amplified flanking sequences are too short to analyze. This could be avoided if one were to use several different pairs of enzymes in order to analyze the samples. It is, thus, highly likely that the incidence of integration of HBV-DNA in a host chromosome is higher than that detected.

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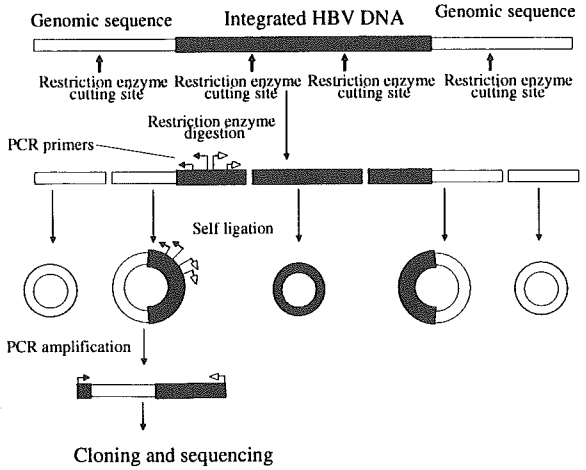

Figure 1 Alu-polymerase chain reaction to detect the integrated hepatitis B virus DNA in host chromosomes.

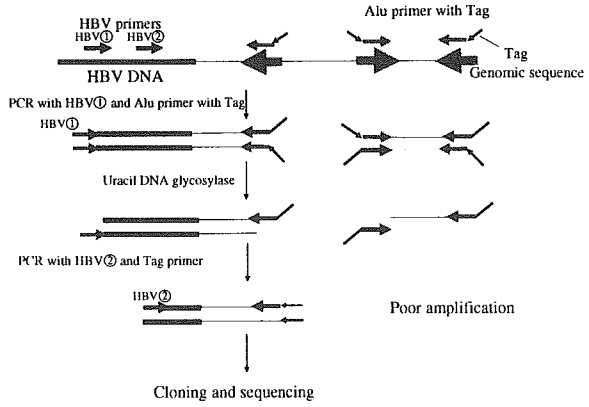

Figure 2 Cellular DNA was amplified using primer HBV1 and Alu specific primer with 5′-Tag sequence. Primers were synthesized using dUTP. The amplified products were then digested with uracil DNA glycosilase. Primers in the amplified DNA were digested with this enzyme leaving complementary sequences. The second round PCR was then carried out using HBV2 and a Tag primer. Only sequences that contain HBV-DNA and Alu sequence were effectively amplified.

An alternative method used to detect integration of HBV-DNA is Alu PCR (Fig. 2).[9] A recent study showed that this method is very sensitive, detecting more than 70% of HCC related to HBV containing integrated HBV-DNA.[10] However, this approach might fail to detect integrated HBV-DNA if the site of integration is too far from the Alu sequences. Also, it is possible that the flanking sequence contains a PCR resistant secondary structure or guanine–cytosine rich regions. Furthermore, similarly to IPCR, if the integrated sequence does not contain a sequence for primers to anneal, the examined integration rate might be underestimated.

The high integration rate (related or unrelated to carcinogenesis) and the fact that the related hepadna virus, the duck hepatitis B virus (DHBV), integrates into a host genome very early after infection, show us that almost all infected cells contain integrated HBV-DNA.[11] However, previous studies have shown that a greater proportion of HBV-DNA integrations are not related to cancer development. Although it is obvious that chronic inflammation and fibrosis are main factors for accelerated cell proliferation and transformation, there are many steps to the development of HCC. Although it remains unclear if the integration of HBV plays an important role in hepatocarcinogenesis, it might open up new avenues of research for hepatocarcinogenesis, for example, to improve the detection of HBV-DNA integration (see Huang *et al.*[6]), and to continue the analysis of the effect of integrated HBV-DNA to intracellular biological phenomenon. Tissues obtained from HCC in children might be good candidates for such analysis because they might cause a serious alteration in chromosomal genetic functions.

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