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Stimulation of hepatocyte survival and suppression of CCl_4-induced liver injury by the adenovirally introduced C/EBPβ gene

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Abstract

Gene therapy has attracted attention as a potentially effective alternative to liver transplantation for the treatment of hepatic failure. We chose the C/EBPβ gene, which plays vital roles in liver regeneration, as a candidate for gene therapy, and examined its effect on hepatocyte survival and the suppression of liver inflammation. C/EBPβ gene overexpression significantly maintained hepatocyte viability during 12 days of the culture. Urea synthesis ability, which is a liver-specific function, in Adv-C/EBPβ-infected hepatocytes was stably maintained during the culture, but the activity per cell was significantly lower than that in non-infected cells. On the contrary, DNA synthesis activity in Adv-C/EBPβ-infected hepatocytes was significantly higher than that in non-infected cells. COX-2 was induced in Adv-C/EBPβ-infected hepatocytes, and the addition of NS398, a specific inhibitor of COX-2, suppressed the viability-maintenance effect. COX-2 was thus shown to be involved in the survival effect of C/EBPβ gene. The introduction of the C/EBPβ gene into liver-damaged mice significantly suppressed the serum AST and ALT activities. These results indicate that C/EBPβ appears to be a survival factor under stressful conditions, and the introduction of the gene has therapeutic function against liver injury.

Keywords: C/EBPβ; COX-2; Adenovirus; Hepatocytes; Gene therapy; Liver injury

Liver transplantation has been successfully established as an effective therapy for both acute and chronic hepatic failure. However, increased demand for the transplantation has caused severe organ shortages all over the world, because the number of organ donors has remained constant. Living donor liver transplantation has also been carried out frequently, but partial hepatectomy of a healthy donor is quite problematic. Therefore, an alternative therapy in place of transplantation should be developed to treat hepatic failure, and thus overcome the problem of organ shortage. Recently, gene therapy has attracted attention as a potentially effective alternative to liver transplantation [1,2]. Fulminant hepatic failure is caused by massive hepatocyte apoptosis and necrosis [3] that can be induced by viral hepatitis, ischemic liver injury, drug toxicity, and other causes. Suppression of hepatocyte death and stimulation of liver regeneration could be an effective treatment for hepatic failure. We have been searching for genes that can confer resistance against apoptotic and necrotic cell death on hepatocytes, and we chose the CCAAT/enhancer-binding protein β (C/EBPβ) gene as a likely candidate for gene therapy.

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C/EBPβ belongs to a family of transcription factors that contain a highly conserved basic-leucine zipper (bZIP) domain at the C-terminus [4]. Six members of the family have been isolated and found to be necessary for normal cellular differentiation and function. C/EBPβ exists in cells as multiple translation products, which are known as the liver-enriched activator protein (LAP) and the liver-enriched inhibitory protein (LIP) [5]. LAP contains both an N-terminal transactivation and a bZIP domain, whereas LIP lacks the activation domain and acts as a dominant-negative inhibitor of transcription. C/EBPβ plays vital roles in liver regeneration [6], adipogenesis [7], and hematopoietic system [8]. We chose to introduce the C/EBPβ gene into hepatocytes because the activated isoform, LAP, appears to be important in promoting proliferation [9]. Here, we show that the adenoviral transfer of the C/EBPβ gene into hepatocytes conferred on them resistance to apoptotic and necrotic cell death and that liver inflammation was suppressed by intraperitoneal administration of the gene.

Materials and methods

Animals. BALB/c mice and Sprague–Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 ± 1 °C prior to the experiment. The experiments were conducted according to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University. Hepatic injury in male mice, aged 6 weeks, was elicited by the intraperitoneal administration of carbon tetrachloride (CCl_4) at 1 ml/kg body weight.

Media. The basal medium consisted of 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B, and 100 ng/ml aprotinin (Nacalai Tesque, Kyoto, Japan) in William's medium E (WE, ICN Biochemicals, Costa Mesa, CA, USA). Medium A consisted of 10% fetal bovine serum (FBS, ICN Biochemicals) in basal medium. Medium B consisted of 1 nM insulin (Sigma Chemicals, St. Louis, MO, USA) and 1 nM dexamethasone (Nacalai Tesque, Kyoto, Japan) in Medium A.

Culture conditions. Hepatocytes were isolated from male Sprague–Dawley rats weighing 150–200 g by perfusing the liver with collagenase (from *Clostridium histolyticum* Type IV; Sigma Chemicals, St. Louis, MO, USA) according to the method of Seglen [10]. Cells were seeded at a density of 1×10^5 cells/cm^2 into 12-well polystyrene culture plates (Nippon Becton–Dickinson, Tokyo, Japan). After 6 h in Medium B, the cells were cultured in Medium A. The medium was changed every 24 h.

Construction of recombinant adenoviral vectors. We constructed an E1- and E3-deleted recombinant adenovirus vector using the pALC3 cosmid, which was generated by removing the E3 region of the adenoviral genome from the pALC cosmid, which already lacks E1 [11]. The C/EBPβ expression cassette, shown in Fig. 1A, was flanked by *Swa*I sites and included the CAG promoter [12], the rat LAP cDNA with sequence starting from the second ATG codon of the full-length C/EBPβ ORF [5], an internal ribosome entry sequence, enhanced green fluorescent protein (EGFP) cDNA, and the rabbit β-globin poly(A) signal. The expression cassette was inserted into the unique *Swa*I site of pALC3, to make pALC3-C/EBPβ. The recombinant adenovirus vector expressing human C/EBPβ (Adv-C/EBPβ) was produced by infecting 293 cells with pALC3-C/EBPβ, and the titer of the virus stock was determined as described previously [11]. Briefly,

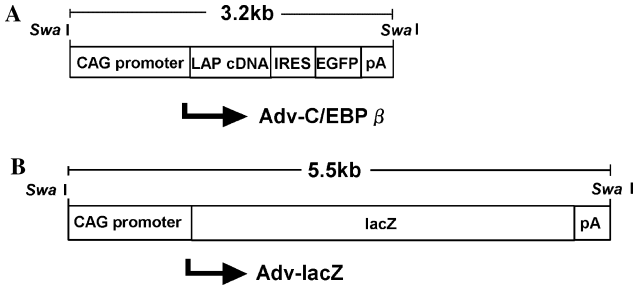

Fig. 1. The expression cassettes were inserted into the pALC3 cosmid. The cassette contained C/EBPβ gene (A) or lacZ gene (B), resulting in Adv-C/EBPβ or Adv-lacZ, respectively.

virus suspensions were serially diluted with medium and added to a 96-well multiplate seeded with 293 cells. After 10 days, the virus titer was calculated by examining the wells for the presence or absence of a cytopathic effect. The control vector, Adv-lacZ, was constructed using the *E. coli* lacZ gene instead of the C/EBPβ gene (Fig. 1B).

Labeling of proliferating cell nuclei with bromodeoxyuridine. Hepatocytes cultured for 6 days were treated with 40 μM bromodeoxyuridine (BrdU) (Nacalai Tesque) for 24 h. The incorporated BrdU was evaluated immunocytochemically. The number of cells with brown nuclei was counted in three randomly selected microscopic fields in each well. The BrdU labeling index (BrdU L.I.) was calculated as the number of BrdU-positive cells/number of cells in the same area $\times 100$ (%).

RT-PCR. The total RNAs were extracted from hepatocytes using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). The gene expression of COX-2 was analyzed using the following primers: forward 5′-TGA TGACTGCCCAACTCCCATG-3′ and reverse 5′-AATGTTGAAG GTGTCCGGCAGC-3′. The gene expression of C/EBPβ was analyzed using the following primers: forward 5′-AAGGCCAAGGCCAAGA AGGC-3′ and reverse 5′-TGAACAAGTTCCGCAGCGTG-3′. β-Actin gene expression was analyzed using the following primers: forward 5′-CATCCCCCAAAGTTCTAC-3′ and reverse 5′-CCAAA GCCTTCATACATC-3′. RT was performed using a 1-μg total RNA sample with the BcaBEST RNA PCR kit (TaKaRa, Kyoto Japan). The PCR conditions were: (1) 94 °C for 1 min; (2) 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and (3) 72 °C for 5 min.

Assays. Adherent cells were treated with trypsin at 37 °C for 5 min, and viability was measured with the trypan blue dye exclusion test. The amount of urea was determined according to the method of Ormsby [13]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an assay kit (IATROZYME TA-Lq; Mitsubishi Kagaku Iatron, Tokyo, Japan).

Statistics. The data were analyzed for statistical significance using Student's *t* test.

Results

Effect of C/EBPβ gene overexpression on hepatocyte function

Isolated rat hepatocytes were infected with recombinant adenovirus during the first 24 h of cultivation on polystyrene culture plates. After the virus was removed, the hepatocytes were cultured in medium A. The translation of the introduced gene was confirmed by observing fluorescence derived from the cotranscribed EGFP gene. Immediately following the 24-h Adv-C/EBPβ

infection period, we observed the fluorescence of the hepatocytes by confocal laser scanning microscopy. Almost all the hepatocytes showed the fluorescence (data not shown). These results indicate that the adenovirally introduced genes were transcribed and then translated within 24 h of hepatocyte culture. Hepatocytes were harvested to analyze the expression of the introduced C/EBPβ gene by RT-PCR. Overexpression of the C/EBPβ gene was observed in the Adv-C/EBPβ-infected hepatocytes at least 8 days of cultivation (data not shown). In contrast, Adv-lacZ-infected and mock-infected hepatocytes stopped expressing endogenous C/EBPβ by the sixth day of culture.

The effect of the C/EBPβ gene introduced by adenoviral infection on the viability of hepatocytes was examined, to learn whether the C/EBPβ gene could potentiate their long-term survival in monolayer culture. Isolated hepatocytes were infected with Adv-C/EBPβ or Adv-lacZ for 24 h; the virus was then removed from the medium and the cells were cultured for the next 11 days. Viable cells were measured by the trypan blue exclusion test at 4, 8, and 12 days of culture. As shown in Fig. 2, Adv-C/EBPβ-infected hepatocytes significantly retained their viability throughout the culture period, as compared with the Adv-lacZ- and mock-infected hepatocytes. Primary cultured hepatocytes on non-coated polystyrene plates tend to die by apoptosis or necrosis and detach from the culture substrate. The overexpression of the C/EBPβ gene appears to prevent this hepatocyte death in the primary culture.

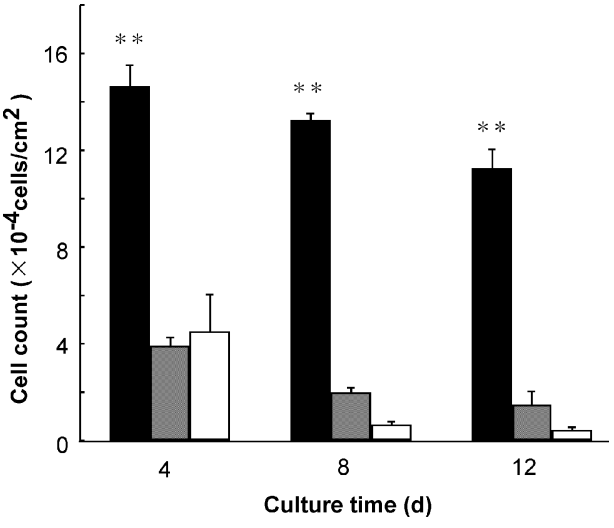

Fig. 2. Maintenance of hepatocyte viability by the Adv-C/EBPβ infection. Hepatocytes were infected with Adv-C/EBPβ at an m.o.i. of 100 (filled bars), Adv-lacZ at a m.o.i. of 100 (gray bars), or mock-infected (open bars). The viability was evaluated with a trypan blue dye exclusion test at the times indicated. Values are means ± SD of three experiments. Asterisks indicate a value significantly different from that of the mock-infected control cells ($p < 0.01$).

The effect of the Adv-C/EBPβ infection on urea synthesis activity, which is a liver-specific function, was examined to learn whether hepatocytes retained this differentiated function under these growth-promoting conditions. Urea synthesis activity was detected and expressed both per unit area and per 10^6 cells. As shown in Fig. 3A, Adv-C/EBPβ-infected hepatocytes retained the activity per unit area during 12 days of cultivation. However, the activity per 10^6 cells was significantly lower than those of the Adv-lacZ- and mock-infected hepatocytes (Fig. 3B). Thus, this function of differentiated hepatocytes appears to be suppressed by the overexpression of the C/EBPβ gene.

As the C/EBPβ gene is well known to be expressed during liver regeneration, we next examined the effect of Adv-C/EBPβ infection on proliferation-related functions in hepatocytes. The uptake of BrdU was measured at 6 days of culture to examine whether the overexpression of C/EBPβ stimulated growth in the hepatocyte monolayers. As shown in Fig. 4, about 35% of the Adv-C/EBP β-infected hepatocytes were positive for BrdU uptake. The percentage of positive cells was significantly higher than that in the cultures of Adv-lacZ- and

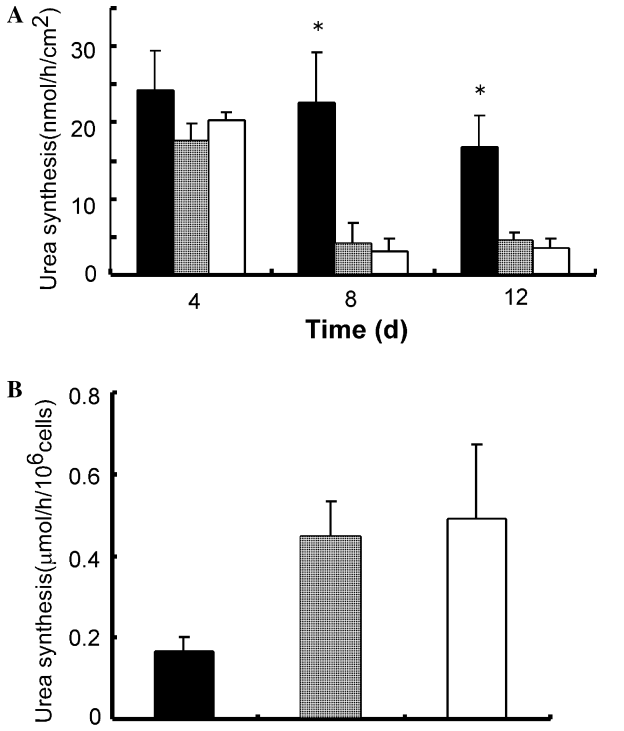

Fig. 3. Effect of Adv-C/EBPβ infection on liver-specific activity in hepatocytes. Urea synthesis activities were measured at the times indicated in hepatocyte cultures that were infected with Adv-C/EBPβ at an m.o.i. of 100 (filled bars), Adv-lacZ at an m.o.i. of 100 (gray bars), or that were mock-infected (open bars). The activity in the upper figure (A) is expressed per unit area. The activity in the lower figure (B) is expressed per 10^6 cells cultured for 4 days. Values are means ± SD of three experiments. Asterisks indicate a value significantly different from that of the mock-infected control cells ($p < 0.05$).

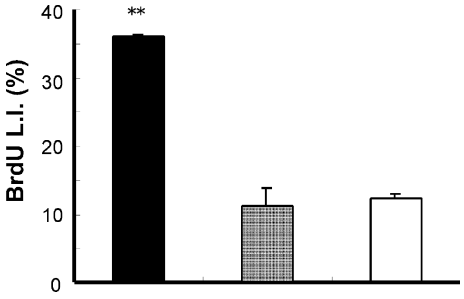

Fig. 4. Effect of Adv-C/EBPβ infection on DNA synthesis in hepatocytes. After 6 days of cultivation, BrdU uptake was measured in hepatocytes infected with Adv-C/EBPβ at an m.o.i. of 100 (filled bars), Adv-lacZ at an m.o.i. of 100 (gray bars), and in mock-infected hepatocytes (open bars). Values are means ± SD of three experiments. The number of BrdU-positive cells/number of cells in the same area × 100 (%) was expressed as BrdU L.I. Asterisks indicates a value significantly different from that of the mock-infected (i.e., m.o.i. of 0) control cells ($p < 0.01$).

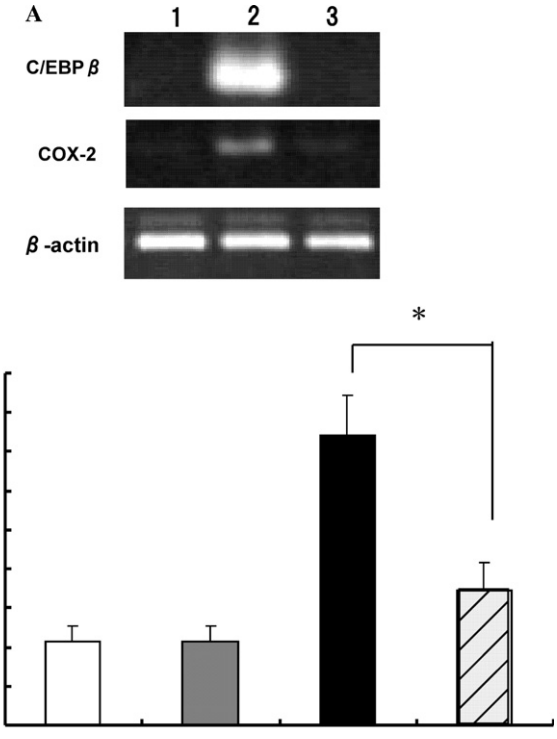

Fig. 5. Involvement of COX-2 gene expression in the effect of the Adv-C/EBPβ infection. Total RNA was extracted from hepatocytes infected with nothing (lane 1), Adv-C/EBPβ at an m.o.i. of 100 (lane 2), or Adv-lacZ at an m.o.i. of 100 (lane 3) at 6 days of cultivation. RT-PCR analysis was performed using the primers for C/EBPβ, COX-2, and β-actin (A). Hepatocytes were cultured for 6 days in the absence (open bar) or presence (gray bar) of NS398 (B). Hepatocytes infected with Adv-C/EBPβ were cultured for 6 days in the absence (filled bar) or presence (hatched bar) of NS398. The viable cells were quantified by the trypan blue dye exclusion test. Values are means ± SD of three experiments. The asterisk indicates a significant difference between the value of viable cells that had been infected with Adv-C/EBPβ and cultured in the absence or presence of NS398 ($p < 0.05$).

mock-infected hepatocytes. Thus, the overexpression of C/EBPβ had a growth-promoting function as well as an anti-apoptotic one. We next examined the involvement of cyclooxygenase-2 (COX-2) induction in the growth-promoting effect of the C/EBPβ gene; COX-2 is well known to contribute to liver regeneration after partial hepatectomy. Total RNAs were extracted from recombinant adenovirus-infected and mock-infected hepatocytes at 6 days of culture. The expression of the COX-2 gene was analyzed by RT-PCR (Fig. 5A). Only Adv-C/EBPβ-infected hepatocytes expressed the COX-2 gene. Hepatocytes were then cultured in the presence of 50 μM NS398, a specific inhibitor of COX-2, to examine the involvement of COX-2 expression in the survival effect of the C/EBPβ gene (Fig. 5B). The addition of NS398 did not affect the viability of the mock-infected hepatocytes. In contrast, NS398 significantly decreased the number of viable hepatocytes infected with Adv-C/EBPβ. These results suggest that the overexpressed C/EBPβ induced the transcription of COX-2, which produced prostaglandins, such as PGE2, that promoted progression of the cell cycle.

Suppression of liver injury

Suppressing hepatocyte death and the subsequent inflammatory response is considered to be important for the prevention and treatment of liver failure. Prior to in vivo experiments, the protective effect of the Adv-C/EBPβ infection on the viability of CCl_4-treated hepatocytes was examined in vitro. After 48 h of cultivation, 10 mM CCl_4 was added and the hepatocytes were cultured for another 4 h. Adv-C/EBPβ-infected hepatocytes had a significantly better survival rate after the CCl_4 treatment (Data not shown). ALT and AST activities in the culture supernatant were elevated by the CCl_4 treatment, but were lower in the cells treated by Adv-C/EBPβ infection. The necrotic cell death induced by CCl_4 was blocked by the overexpressed C/EBPβ gene.

We next used in vivo experiments to examine the therapeutic effects of C/EBPβ gene overexpression in mice injured by CCl_4. Intraperitoneal administration of CCl_4 to male BALB/c mice was conducted to induce liver injury. Six hours after the CCl_4 treatment, 1×10^9 *pfu* of Adv-C/EBPβ or Adv-lacZ was administered intraperitoneally to the injured mice. Serum AST and ALT activities were measured 48 h after the CCl_4 injection (Fig. 6). The normal activities of AST and ALT in the sera from untreated mice were 65 ± 6 and 27 ± 4 (KU), respectively. The serum AST and ALT activities in injured mice were elevated to 3042 ± 310 and 2829 ± 298, respectively. There was no significant change in the serum AST and ALT activities between Adv-lacZ- and mock-infected mice. In contrast, the serum AST and ALT activities were significantly lower in the Adv-C/EBPβ-infected mice. These results indicate that the C/EBPβ gene introduction has a therapeutic

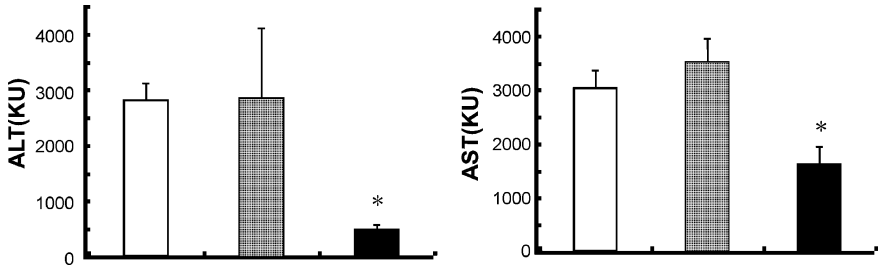

Fig. 6. Suppression of liver inflammation by Adv-C/EBPβ administration. BALB/c mice were intraperitoneally injected with CCl_4, and then saline (open bar), Adv-lacZ (gray bar), or Adv-C/EBPβ (filled bar) was also later given by intraperitoneal injection ($n = 3$). After 48 h, the ALT and AST activities in the serum were measured. Values are means ± SD of three experiments. The asterisk indicates a value significantly different from that of the mock-infected control mice ($p < 0.05$).

function against hepatocyte injury and suggest that this gene could be used to treat patients with hepatic failure.

Discussion

The C/EBPβ gene, which is upregulated during liver regeneration, was chosen as a candidate gene for conferring on hepatocytes resistance to apoptotic and necrotic cell death. An adenovirus vector was used in this study because of its high gene transduction efficiency and its rapid accumulation in the liver from the bloodstream. The endogenous expression of the C/EBPβ gene in cultured hepatocytes gradually decreased and was almost lost by 6 days of cultivation. The viability of the hepatocytes significantly improved with increased expression levels of the C/EBPβ gene. The introduction of the C/EBPβ gene by adenovirus infection markedly increased the number of viable hepatocytes during the 12 days of culture. Liver-specific activities and growth-related functions are reported to be reversely regulated in hepatocytes [14,15]. We found that the urea synthesis activity in the Adv-C/EBPβ-infected hepatocytes was maintained, but at a significantly lower level than in the control cells. Thus, this function of differentiated cells was suppressed, and DNA synthesis activity was upregulated by the overexpression of the C/EBPβ gene. In our previous study, stress-responsive genes encoding heat shock proteins were upregulated in monolayer cultures of hepatocytes [16]. Thus, the C/EBPβ protein appears to be a hepatocyte survival factor that is upregulated under stressful conditions. In this study, we also showed that Adv-C/EBPβ infection conferred on hepatocytes resistance to CCl_4 injury. Therefore, gene therapy using the C/EBPβ gene to enhance hepatocyte survival may be effective for treating acute liver failure in humans.

The induction of COX-2 was involved in the enhancement of hepatocyte survival by the overexpression of the C/EBPβ gene. COX-2 is induced during liver regeneration after partial hepatectomy, and its induction is concomitant with an increase in the expression of C/EBPβ [17,18]. It has been reported that C/EBPβ is essential for COX-2 gene transcription in articular chondrocytes and macrophages [19,20]. The induction of COX-2 mediated by overexpression of the C/EBPβ gene could result in the production of prostaglandins, promoting the cell cycle, and conferring on the hepatocytes resistance to apoptotic and/or necrotic cell death under stressful conditions that would normally kill them. Since COX-2 is also upregulated in many cancer cells [21–23], the persistent expression of the COX-2 gene seems undesirable, because of the possible risk of cancer development. However, the expression of genes cloned into the adenovirus vector is transient, maintained at most for 2–3 weeks, and the subsequent COX-2 induction is not expected to last long enough for cancer to develop.

We have shown that the administration of Adv-C/EBPβ to CCl_4-injured mice suppressed liver inflammation. Because there was no significant difference in the amount of serum AST and ALT of the mock- and Adv-lacZ-infected mice, the adenovirus infection appears to be non-toxic under the conditions used in this study. Here, we used a mutant of adenovirus type 5 (Ad5) that has deletions in the E1 and E3 regions [11]. It has been reported that Ad5 is immediately cleared from the bloodstream and accumulates in the liver, where it is mainly taken up by hepatocytes and Kupffer cells [24,25]. In our preliminary experiments, we examined whether Adv-C/EBPβ infected hepatocytes in vivo following intraperitoneal administration. Hepatocytes were isolated 24 h after the injection and cultured in a monolayer on polystyrene plates. Fluorescence derived from the expression of the co-transcribed EGFP gene was observed in some hepatocytes, by confocal laser scanning microscopy (data not shown). It is not clear how much Adv-C/EBPβ is trapped in Kupffer cells or whether the introduced gene in the Kupffer cells contributes to suppression of the CCl_4-induced liver inflammation. Further analysis and improvement of the adenovirus vectors are necessary to efficiently deliver the gene into hepatocytes.

Development of therapies based on the suppression of hepatocellular apoptosis and necrosis to prevent liver failure is necessary so as to create an alternative to liver

transplantation. In this study, we found that C/EBPβ could function as a survival factor for hepatocytes and that overexpression of the C/EBPβ gene was remarkably effective for protecting hepatocytes from injury. Since the administration of Adv-C/EBPβ after the liver was injured with CCl_4 significantly suppressed liver inflammation, gene transduction may be useful therapeutically for patients with acute and chronic hepatic failure.

Acknowledgments

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