

Oltipraz regenerates cirrhotic liver through CCAAT/enhancer binding protein-mediated stellate cell inactivation

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ABSTRACT

Liver cirrhosis (LC) is a chronic disease with high mortality rate. In the United States and Western world as well as Asian countries, LC is the major leading cause of death by disease. Yet, no effective therapeutic agent is available for LC treatment. Laboratory cirrhotic rats produced by dimethylnitrosamine administrations simulate the clinical features of human LC such as mortality, ascites, hepatic parenchymal cell destruction, and formation of connective tissue and nodular regeneration, providing a preclinical model to evaluate therapeutic efficacy of drugs and the underlying mechanisms. Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] has been used clinically and is of little toxicity. Comprehensive mechanistic and phase IIa clinical studies supported the notion that oltipraz exerts chemopreventive effects against chemical carcinogenesis. We report here that oltipraz within the clinical dose range regenerates cirrhotic liver in the established LC rats as a result of reduction of the intensities of cirrhotic nodules, elimination of accumulated extracellular matrix, and inactivation of stellate cells, thereby improving survival rate. We also reveal that activation of CCAAT/enhancer binding protein by oltipraz inhibits transforming growth factor β 1 gene expression in stellate cells, which provides a molecular target for pharmacological treatment of LC. Oltipraz is the first therapeutic agent that regenerates cirrhotic liver.

Key words: liver cirrhosis • C/EBP • TGF- β 1

Liver cirrhosis (LC) is best defined as a chronic liver disease with a high mortality rate. Chronic alcoholism and hepatitis C virus are the primary causes of cirrhosis in the United States and Western world, whereas hepatitis B virus is the most common cause of cirrhosis worldwide. The incidence of cirrhosis is 10–20% among chronic alcoholics. Infection with hepatitis C virus accounts for 40% of the end-stage cirrhotic cases (1). In areas where hepatitis B virus infection is endemic (e.g., southeast Asia, sub-Saharan Africa, and other Asian countries), up to 15% of the population may be infected with the virus during childhood,

and cirrhosis may develop in one-fourth of these chronic carriers. In the United States, LC is the tenth leading cause of death by disease, with 373,000 individuals hospitalized yearly and 25,000 deaths annually (2). In Japan, the death from LC is the ninth leading cause of death, with 16,000 deaths annually (3). In addition, there is also a great toll of human suffering, hospital costs, and job loss by people with cirrhosis.

Pathogenesis of LC includes hepatic parenchymal cell destruction, formation of connective tissue, and nodular regeneration. Therapeutic management of LC is primarily symptomatic, directed at clinical manifestations such as ascites, esophageal varices, and hepatic encephalopathy. A few drugs, including ursodesoxycholic acid, silymarin, and lamivudine have been used for patients with LC. Although ursodesoxycholic acid is the only approved treatment for primary biliary cirrhosis and has shown partial benefits in some patients (4, 5), published randomized controlled trials of the drug did not show evidence of therapeutic benefit (6, 7). Silymarin, despite its protective effects in different experimental conditions, has no effect on survival and the clinical course in LC (8). Lamivudine, an antiviral agent, improves some hepatic function in patients with decompensated cirrhosis and replicating hepatitis B virus (9), but the efficacy of lamivudine for the treatment of LC is still uncertain. Unfortunately, no agent effective for regeneration of cirrhotic liver is currently available. It has been proposed that repeated transduction of skeletal muscles with hepatocyte growth factor gene is potentially useful for the treatment of LC in an animal model (10). Due to problems in targeted DNA delivery, the duration of transgene expression, and adverse consequences of heterologous gene expression, the approach of gene therapy is limited.

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] has been extensively studied as a cancer chemopreventive agent (11–14). Phase IIa human clinical studies of oltipraz on cancer chemoprevention, conducted in Qidong, China, showed that the drug might be active as a cancer chemopreventive agent (15, 16). Comprehensive mechanistic studies supported the notion that oltipraz exerts chemopreventive effects against chemical carcinogenesis through the induction of phase II enzymes (14) and the intervention of cytochrome P450-catalyzed metabolic activation (17, 18).

Hepatic fibrosis, which arises from overproduction of extracellular matrix, including collagens, is a prepathologic state of cirrhosis that occurs as a consequence of severe liver damage in diverse chronic liver diseases. Fibrogenesis with loss of liver function leads to development of cirrhosis (19). Dimethylnitrosamine (DMN) through metabolic activation by cytochrome P450 2E1 exerts hepatotoxicity and tissue injury (20, 21). Liver injuries induced by multiple DMN treatments lead to hepatic necrosis, fibrosis, and eventually cirrhosis (22). Recently, we reported that oltipraz prevented DMN-induced tissue destruction and subsequent replacement by connective tissue (23).

Hepatic stellate cells cause synthesis of large quantities of extracellular matrix (24). Transforming growth factor β 1 (TGF- β 1), as a key fibrogenic mediator for fibrogenesis after injuries through deposition of extracellular matrix and inhibition of collagenase activity in the liver, is associated with the regulation of cell growth and differentiation and causes synthesis of extracellular matrix proteins and cellular receptors for matrix proteins (24).

In view of the previous observation that oltipraz prevented liver fibrogenesis, we were challenged to study the effectiveness of oltipraz in regeneration of cirrhotic liver. We now report the efficacy of oltipraz, which includes reduction of the intensities of liver fibrotic and cirrhotic nodules, elimination of accumulated extracellular matrix, and regeneration of cirrhotic liver. Oltipraz completely resolves fibrosis in the cirrhotic liver, thereby improving viability. TGF- β 1 signaling plays an important role in liver fibrogenesis and cirrhosis as evidenced by receptor knockout experiments (25). No therapeutic agent that is active in interrupting TGF- β 1 signaling is available. To render investigations on the therapeutic basis of oltipraz, we hypothesized that oltipraz inhibits TGF- β 1 expression in stellate cells via activation of CCAAT/enhancer binding protein (C/EBP). We propose that C/EBP serve as a molecular target for the treatment of LC.

MATERIALS AND METHODS

Oltipraz was provided from Aventis Pharma France (Vitry-sur-Seine, France). Reverse transcriptase and *Taq*. polymerase were obtained from Promega (Madison, WI) and Clontech (Palo Alto, CA), respectively. Fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody was obtained from Zymed Laboratories (San Francisco, CA). DMN and other reagents were purchased from Sigma (St. Louis, MO).

Animals

Sprague-Dawley rats at 6 wk of age (140–160 g) were used. To induce LC, DMN dissolved in sterile saline was i.p. injected (10 μ l/kg) to rats three times per week for 3 wk, and then on the fourth week, the rats were subjected to three consecutive daily DMN injections and housed for 5 days without further treatment. A large fraction of rats showed the characteristic pathological changes found in LC after DMN administrations, having body weight of <250 g. Cirrhotic rats were randomly distributed to three groups ($n=18$ per treatment group). To cirrhotic rats, oltipraz (Aventis) suspended in 40% polyethylene glycol 400 was orally administered (15 or 30 mg/kg, three times per week for 4 wk). Control cirrhotic animals received vehicle only. We verified that the vehicle had no effect on the liver. Untreated healthy animals were used as control rats. The mortality and body weight were monitored during the 4 wk of treatment. Surviving animals were sacrificed on day 28. The liver was excised, weighed, and subjected to histopathological examinations and subcellular fractionation. Whole brain and kidney were excised and weighed for comparative purposes.

Blood chemistry

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, bilirubin, total proteins, and albumin in plasma were analyzed using Spectrum, an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL).

Representative hematology

Hematologic evaluations consisted of white blood cell (WBC) count, red blood cell (RBC) count, platelet count, and hemoglobin concentration. Hematology was monitored using Hemavet 850 (CDC Technologies, Oxford, CT).

Histopathology

Hepatic morphology was assessed by light microscopy. The left lateral lobe of the liver was sliced (three slices per rat), and tissue slices were fixed in 10% buffered-neutral formalin for 6 h. Fixed liver tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, UK). Sections of 4 μm in thickness were subjected to Masson's trichrome staining before examinations (26). A certified pathologist scored samples in a blinded fashion. An arbitrary score was given to each microscopic field viewed at a magnification of 100 \times . A minimum of 10 fields was scored per liver slice to obtain the mean value. Extents of periportal bridging, intralobular degeneration, portal inflammation, and fibrosis were also graded according to the Knodell's scoring method (27).

Immunohistochemistry of type I collagen and α -smooth muscle actin (α -SMA)

Immunochemical analyses for type I collagen and α -SMA were performed using the respective specific antibodies (anti-type I collagen antibody, Santa Cruz Biotechnology [Santa Cruz CA]; anti- α -SMA antibody, Neomarkers [Fremont, CA]). We used a commercially available indirect avidin-biotin-alkaline phosphatase kit (InnoGenex, San Ramon, CA) for immunohistochemistry. In brief, tissue sections on slides were deparaffinized, hydrated at room temperature, and blocked with serum. Sections were then incubated with the respective antibody for 30 min at room temperature in a humidified chamber. Slides were washed 3–5 times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 after incubation with antibody. Sections were then incubated with appropriate biotinylated secondary antibody for 20 min at 37°C, followed by streptavidin-alkaline peroxidase complex for 5 min at 37°C. Slides were washed with PBS three to five times after each incubation. Samples on slides were incubated with 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium as substrates for phosphatase reaction until adequate color was developed. For some experiments, the sections were counterstained with nuclear Fast Red.

Cell culture

Hepatic stellate cells were isolated from male Sprague-Dawley rats according to a previously published method, with a slight modification (28). Rat livers were perfused *in situ* with Ca^{2+} -free Hank's balanced saline solution (HBSS) at 37°C for 5 min. Livers were then perfused for 20 min with HBSS containing 0.05% collagenase and Ca^{2+} at a perfusion flow rate of 10 ml/min. Room temperature was kept above 35°C during liver perfusion. After perfusion, the livers were minced gently with scissors and suspended with sterilized PBS. The cell suspension was then filtered through sterilized gauze and centrifuged at 50g for 5 min to separate parenchymal and nonparenchymal cells. The supernatant was further centrifuged at 500g for 10 min. After centrifugation, the pellet was resuspended in 8.2% Nycodenz (Sigma) and centrifuged at 1400g for 15 min. Cells were collected from the interface between the top layer of PBS and the Nycodenz. The cells showed typical autofluorescence due to vitamin A-rich fat droplets. Hepatic stellate cells were cultured on plastic dishes. Both types of cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin/streptomycin (100 $\mu\text{g}/\text{l}$) supplemented with 10% fetal bovine serum.

Immunoblot analyses of α -SMA and proliferating cell nuclear antigen (PCNA)

Samples for detection of α -SMA were prepared from the liver homogenates in 0.1 M Tris-acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA by centrifugation at 10,000g for 30 min and stored at -70°C until use. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to Laemmli (29). Immunoblot analysis was performed according to a previously published procedure (30). Immunoblot analysis for PCNA was carried out with nuclear extracts, the fraction of which was obtained by a modification of the published procedure (31). The samples were fractionated by 12% for α -SMA and PCNA gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with monoclonal mouse anti- α -SMA antibody (1:2000) (Neomarkers, Fremont, CA) or polyclonal mouse anti-PCNA antibody (1:1000) (Santa Cruz Biotechnology), followed by incubation with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody. Then it was developed using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit (30, 32).

Immunocytochemistry of C/EBP β

Activated stellate cells were grown on Lab-TEK chamber slides (Nalge Nunc International, Rochester, NY) and incubated in DMEM containing 10% fetal bovine serum. The standard immunocytochemical method was used as described previously (33). For immunostaining, cells were fixed in 100% methanol for 30 min and washed three times with PBS. After blocking in 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature, the cells were incubated for 1 h with anti-C/EBP β antibody (1:100) in PBS containing 5% BSA. The cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:100) after serial washings with PBS. The location and integrity of nuclei were verified by counterstaining with propidium iodide (2 $\mu\text{g}/\text{ml}$). Stained cells were washed and examined using a laser scanning confocal microscope (Leica TCS NT, Leica Microsystems, Wetzlar, Germany).

Decoy oligodeoxynucleotides technique

Double-stranded oligodeoxynucleotides (ODN) were prepared from complementary single-stranded phosphorothioate-bonded ODN (Bioneer, Chungbuk, Korea) by melting at 95°C for 5 min followed by a cool down phase at ambient temperature. The sequences of the single-stranded ODN were as follows (underlined letters denote phosphorothioate-bonded bases): C/EBP, 5'-TGCAGATTGCGCAATCTGCA-3'; mutant C/EBP, 5'-TGCAGAGACTAGTCTCTGCA-3' (34). Decoy ODN were preincubated for 1 h in stellate cells and then further incubated with or without 30 μM oltipraz for 12 h. Transfection of the decoy ODN (dODN) was achieved without using a cationic lipid or liposomal complex.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR

RT-PCR was performed using the selective primers for TGF- β 1 (sense primer: 5'-CTTCAGCTCCACAGAGAAGAAGACTGC-3', antisense primer: 5'-

CACGATCATGTTGGACAACCTGCTCC-3')(298 bp), procollagen α 1(III) (sense primer: 5'-AATTCTGCCACCCTGAACTC-3', antisense primer: 5'-GTATTGGTGGGTGAAACAGC-3')(784 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(sense: 5'-TCGTGGAGTCTACTGGCGT-3', antisense: 5'-GCCTGCTTCACCACCTTCT-3')(510 bp) genes. PCRs were carried out for 34 cycles using the following conditions: denaturation at 94°C for 0.5 min, annealing at 49°C for 0.5 min, and elongation at 68°C for 1 min. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator. The SYBR Green real-time RT-PCR amplifications were undertaken in a 96-well GeneAmp PCR System 9700 coupled with a ABI Prism 7000 Sequence Detection System (AB Applied Biosystems, Alameda, CA). The TGF- β 1 amplifications were carried out in a 20- μ l reaction volume containing 10 μ l of 2 \times SYBR Green master mix, 0.1 μ M each of forward and reverse primers, and 8 μ l of the diluted cDNA. The C_T values of GAPDH amplification were obtained by using serially diluted cDNA samples, and the samples with the same C_T values were used for equal loading of cDNA. Thermal profile for SYBR Green RT-PCR was 95°C for 3 min following 45 cycles at 95°C for 20 s, at 49°C for 30 s, and at 72°C for 1 min.

Statistical analysis

The χ^2 test was used to assess the statistical significance of the survival rates of cirrhotic rats. Paired Student's *t* test was used to determine the significance in the differences between two group means. One-way ANOVA was used to assess significant differences among treatment groups. Data represent the mean \pm SE. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

RESULTS

Oltipraz increases the survival rates of cirrhotic rats as a result of improvement of liver function and regeneration of cirrhotic liver

We first induced LC in rats by injecting multiple doses of DMN for 4 wk and then determined whether the survival rate of cirrhotic rats improved during the next 4 wk of oltipraz treatment ([Fig. 1](#)). A large fraction of vehicle-treated cirrhotic rats died within the first 3 wk. Oltipraz treatment (30 mg/kg, three times per week, orally for 4 wk) improved the survival rate ($P < 0.05$; χ^2 test) of these rats to 82% on day 28 compared with 47% in vehicle-treated cirrhotic animals ([Fig. 1](#)). Rats treated with oltipraz (30 mg/kg) had significantly greater body weight gain on day 28 than that of vehicle-treated ones (38% vs. 25%; $P < 0.05$, paired Student's *t* test) ([Fig. 1](#), inset).

In LC, a decrease in plasma oncotic pressure as a result of impaired albumin synthesis in combination with elevated hydrostatic pressure causes accumulation of ascitic fluid in the peritoneal cavity (35). Fluid accumulation in the peritoneal cavity was indexed 4 wk after the treatment of cirrhotic rats with oltipraz. Whereas the ascitic index of cirrhotic rats was 1.63 ± 0.42 , oltipraz treatment at the dose of 30 mg/kg significantly reduced that to 0.36 ± 0.16 ([Fig. 2A](#)). The level of plasma albumin is regarded as an important index of liver function. The decreased synthesis of albumin in the liver accompanies edema and ascites formation. The plasma albumin, which was significantly decreased to 76% of control (3.23 ± 0.21 g/dl) in

cirrhotic rats, was restored by oltipraz at the dose of 30 mg/kg to 90% of healthy control animals (3.81 ± 0.18 g/dl) ([Fig. 2B](#)).

To determine whether oltipraz had an impact on liver regeneration, weights of the major organs excised after drug treatment were measured. In contrast to no or minimal changes in brain and kidney weights, liver weight in cirrhotic rats was decreased to 55% of that in healthy controls. The liver-to-brain weight ratio in oltipraz-treated rats significantly increased up to 93% of that in control ([Fig. 2C](#)). PCNA is expressed in dividing cells in late G1 and S phases and used as a marker for cell proliferation (36). Western blot analysis showed an increase in the band intensity of 36-kDa PCNA in the liver homogenates of cirrhotic rats treated with oltipraz, as compared with either healthy control or vehicle-treated cirrhotic animals ([Fig. 2D](#)). The increase in PCNA in conjunction with the increase in the liver-to-brain weight ratio supports the notion that oltipraz regenerates cirrhotic liver.

In addition, we determined the effect of oltipraz on the maturation of liver stem cells in cirrhotic rats. Thy1.1- or Flt-3-positive undifferentiated cells were not detected in healthy control liver. In contrast, Thy1.1- or Flt-3-positive cells were detected in proximity to portal spaces of liver lobules in cirrhotic rats (28 ± 4 and 27 ± 5 /arbitrary visual field, respectively; $n=7$). The number of Thy1.1- or Flt-3-positive cells in cirrhotic liver was significantly decreased by oltipraz treatment (8 ± 1 and 6 ± 2 /arbitrary visual field, respectively; significant compared with cirrhotic rats, $*P<0.05$; $n=7$), which was consistent with the expected functional improvements in hepatocyte maturation and differentiation by oltipraz.

In the present study, ALT and AST activities were elevated to certain extents in cirrhotic rats ([Table 1](#)). The plasma AST activity was significantly decreased by 67% with oltipraz treatment (30 mg/kg).

Oltipraz reduces the intensities of liver cirrhotic nodules and eliminates accumulated extracellular matrix

To determine whether cirrhosis could be treated with oltipraz, we histopathologically examined the formation of cirrhotic nodules, extent of liver fibrosis, intralobular hepatocyte degeneration, and portal inflammation of surviving cirrhotic rats after 4 wk of vehicle or drug treatment. Healthy control rats showed no pathological changes ([Fig. 3A-a](#)). Masson's trichrome staining revealed that extracellular matrix was heavily accumulated around and within thick multiple fibrotic nodules, particularly in proximity to portal spaces in the liver of cirrhotic rats ([Fig. 3A-b](#)). Treatment of cirrhotic rats with 15 mg/kg of oltipraz for 4 wk notably decreased the intensities of liver fibrotic nodules ([Fig. 3A-c](#)). Furthermore, liver fibrotic nodules completely disappeared after 30 mg/kg of oltipraz treatment. Only marginal fibrous bands were detected ([Fig. 3A-d](#)). We quantitated the extents of fibrosis in cirrhotic rats. In cirrhotic animals treated with 30 mg/kg oltipraz, the fibrosis scores significantly decreased ([Fig. 3B](#), left). By multiple analyses, we obtained Knodell scores, which were also significantly decreased by oltipraz treatment ([Fig. 3B](#), right). Immunohistochemical analysis showed that the expression of type I collagen, a representative collagen, was markedly elevated in cirrhotic liver. Type I collagen accumulated in cirrhotic liver disappeared after oltipraz treatment ([Fig. 3C](#)).

Oltipraz inactivates stellate cells in cirrhotic liver

As a second approach to investigate whether oltipraz can treat LC, we sought to determine the number of activated stellate cells in cirrhotic liver by immunostaining α -SMA, a definitive marker. No α -SMA-positive cells were detected in the liver of healthy control rat (Fig. 4A-a). As expected, α -SMA-positive cells multiplied and stained as single cells or clusters lined within or in the vicinity of accumulated fibers (Fig. 4A-b). Duplicate samples stained with Masson's trichrome showed accumulated fibers (Fig. 4A-b). In cirrhotic rats treated with oltipraz (30 mg/kg, 4 wk), α -SMA-positive cells were rarely detected (Fig. 4A-c). Western blot analysis was used to confirm the expression of α -SMA. Whereas control rats showed no α -SMA expression, the expression of α -SMA in cirrhotic rats substantially increased (Fig. 4B). Consistent with immunohistochemistry, the level of α -SMA in liver homogenates was almost completely decreased with oltipraz treatment (30 mg/kg, three times per week, 4 wk) (Fig. 4B). Desmin, a marker protein expressed primarily in quiescent stellate cells, was stained for comparison. The expression of desmin was decreased in the liver of cirrhotic rats (Fig. 4B) (37, 38). Actin immunoblot verified equal loading of proteins. The expression of TGF- β 1 mRNA was assessed by RT-PCR, which is adequate for detection of TGF- β 1 expression (39). The production of TGF- β 1 mRNA, which was increased in cirrhotic liver, was inhibited by oltipraz treatment (Fig. 4C).

The pharmacological molecular targets for the inactivation of activated stellate cells include C/EBP

To determine whether oltipraz inhibited transactivation of hepatic stellate cells and promoted stellate cell inactivation, the expression of α -SMA and TGF- β 1 was measured in cultured stellate cells exposed to oltipraz *in vitro*. Pharmacokinetic and tissue distribution studies showed that the areas under the plasma concentration-time curve from time zero to time infinity of oltipraz increased proportionally to an increase in oral doses and that the hepatic drug concentration in healthy control or cirrhotic rats treated with a dose of 30 mg/kg was 23–27 μ M (S.G. Kim and M.G. Lee, unpublished data). Thus, we chose 3, 10, or 30 μ M oltipraz for *in vitro* experiments. The induction of α -SMA was notably inhibited by oltipraz at 3–10 μ M for 1 day or 3 days (Fig. 5A). Hepatic stellate cells synthesize large quantities of extracellular matrix (24). TGF- β 1 as a key fibrogenic mediator causes synthesis of extracellular matrix proteins and inhibits collagenase activity in the liver (24). We reported that the expression of TGF- β 1 in the whole liver was decreased by oltipraz pretreatment (23). TGF- β 1 gradually increases during stellate cell activation (40). We next determined whether oltipraz directly inhibits TGF- β 1 expression in activated stellate cells. The level of TGF- β 1 mRNA was decreased by the presence of 3–30 μ M oltipraz, whereas that of GAPDH mRNA was not changed (Fig. 5B). Consistent with the inhibition of TGF- β 1, oltipraz decreased the expression of the procollagen α 1(III) gene in activated stellate cells (Fig. 5B).

Studies in our laboratory revealed that oltipraz induced the nuclear translocation and activation of C/EBP β and stimulated C/EBP-mediated gene expression in H4IIE rat hepatoma cells (Kang et al., unpublished results). Putative C/EBP response elements are present in the 5'-flanking region of the rat *TGF- β 1* gene (41). Given the activation of C/EBP β by oltipraz and the presence

of C/EBP response element in the *TGF-β1* gene, we assessed the role of C/EBPβ in the *TGF-β1* gene expression in stellate cells. Immunocytochemistry revealed that oltipraz (30 μM) caused cytosolic C/EBPβ to translocate into the nucleus in activated stellate cells (Fig. 5C), which was confirmed by Western blot analysis (data not shown). Because the transfection efficiency of dominant negative mutant of C/EBP (AC/EBP) to activated stellate cells was too low (i.e., 5–20%), we could not assess whether AC/EBP reversed the effect of oltipraz. Hence, we used C/EBP-specific decoy oligodeoxynucleotides (dODN) to determine the role of C/EBPβ in the suppression of *TGF-β1* gene. RT-PCR analysis revealed that oltipraz inhibited TGF-β1 expression in cells treated with mutant C/EBP dODN (Fig. 5D, upper). A preliminary study showed that C/EBP negatively regulates TGF-β1 expression (S.G. Kim and M.K. Cho, unpublished data). In the present study, C/EBP dODN enhanced the expression of TGF-β1 in control cells as a result of blocking the negative regulation of TGF-β1 expression by C/EBP. In stellate cells treated with oltipraz, C/EBP dODN reversed the inhibition of TGF-β1 mRNA by oltipraz (Fig. 5D, upper). SYBR Green real-time RT-PCR analysis was used to further confirm the changes in the TGF-β1 mRNA in stellate cells (Fig. 5D, lower). The level of TGF-β1 mRNA was suppressed by oltipraz in cells exposed to mutant C/EBP dODN, as evidenced by a decrease in C_T value ($\Delta C_T=2.2$). In cells pretreated with C/EBP dODN, oltipraz failed to decrease the level of TGF-β1 mRNA ($\Delta C_T=-2.4$). Thus, we conclude that the inhibition of extracellular matrix accumulation by oltipraz in the liver of cirrhotic rats may result from C/EBPβ-mediated TGF-β1 suppression in stellate cells.

DISCUSSION

Laboratory cirrhotic rats produced by DMN administrations simulate the clinical features of human LC such as mortality, ascites, hepatic parenchymal cell destruction, formation of connective tissue, and nodular regeneration, providing a preclinical model to evaluate therapeutic efficacy of drug and underlying mechanism. Deposition of extracellular matrix components, including collagen is a common phenomenon in LC. We showed for the first time that oltipraz effectively treats LC in rats with improvement of liver function. Treatment of cirrhotic rats with oltipraz caused a decrease in accumulation of extracellular matrix. Oltipraz markedly reduced the number of cirrhotic nodules and the staining intensities of nodular capsules. Fibrosis index and type I collagen accumulation were both significantly decreased by 4 wk of oltipraz treatment in cirrhotic rats. Anticirrhotic effects were further supported by decreases in Knodell score, a general marker of LC and inflammation (27).

In cirrhotic rats, functional disturbances in the liver were supported by accumulation of peritoneal fluid as a result of a decrease in plasma albumin content and presumable fibrosis-induced portal hypertension. The elevated ascites index in cirrhosis was decreased by oltipraz by ~80%. The decreased synthesis of albumin, which resulted in edema and ascites formation in LC, was restored by oltipraz treatment to 90% of healthy control. Hence, oltipraz improved liver function. We also monitored other biochemical parameters for liver function. The liver transaminase activity represents increased leakage from injured hepatocytes rather than from dead cells, and this does not correlate quantitatively well with the liver dysfunction as a result of cirrhosis (42). However, the plasma transaminase activity is increased with biliary obstruction in cirrhotic patients (43). In the present study, the plasma AST activity in cirrhosis was decreased

by ~30% with oltipraz treatment. More importantly, oltipraz significantly increased liver weight in cirrhotic rats almost to that of healthy controls. The increase in PCNA by oltipraz further supports the notion that oltipraz regenerates cirrhotic liver by liver cell proliferation. This is also consistent with maturation of undifferentiated cells in cirrhotic livers by oltipraz.

In view of the importance of C/EBPs as targets for the regulation of liver regeneration, we assessed the levels of nuclear C/EBPs. The level of nuclear C/EBP β (liver-activating protein) was down-regulated in LC, whereas oltipraz treatment increased that of C/EBP β in cirrhotic rats (data not shown). Conversely, the truncated isoform of C/EBP β (liver-enriched inhibitory protein), whose expression remained elevated in LC, was completely suppressed by oltipraz. LC caused the level of nuclear C/EBP α , the predominant C/EBP isoform expressed by adult hepatocytes in healthy livers, to be markedly suppressed. The level of C/EBP α was partially restored by oltipraz. Oltipraz was also capable of directly activating C/EBP α and C/EBP β in cultured hepatocytes (S.G. Kim, unpublished data).

It has been reported that malotilate, a clinical liver protective agent, was cytotoxic to peripheral blood mononuclear cells, particularly to those from patients with chronic active hepatitis (44). In contrast, oltipraz did not show notable blood toxicity. In the present study, the effect of oltipraz on the representative hematology was assessed in cirrhotic rats. Although the hematological parameters, including RBC, hemoglobin, WBC, and platelet, were decreased to a certain extent in cirrhotic rats, the changes were not statistically significant irrespective of oltipraz treatment (data not shown). The result of blood chemistry provided the evidence that oltipraz at the doses used did not affect hematology.

Activation of stellate cells in cirrhotic animal and in culture involves major changes in phenotype, including increased expression of α -SMA. Thus, α -SMA is used as a definite marker of transdifferentiation of stellate cells (45). Oltipraz actively inhibited the number of α -SMA-positive cells and the production of α -SMA in cirrhotic livers. To further delineate whether the inhibition of α -SMA production by oltipraz was the direct effect, an additional study was conducted in cultured stellate cells. α -SMA production was significantly inhibited by oltipraz at concentrations of 3–10 μ M in activated stellate cells. This indicates that oltipraz antagonizes transdifferentiation of stellate cells in both cirrhotic liver and cultured cells.

Cytokines would constitute the basis for mesenchymal cells in liver fibrogenesis. Stellate cells are the predominant source of the extracellular matrix proteins characterizing hepatic cirrhosis (46). Transactivation of quiescent stellate cells to activated ones plays an essential role in fibrogenesis in the liver after injury. Whereas quiescent hepatic stellate cells produce hepatocyte growth factor, activated stellate cells are involved in the production of TGF- β 1 (47, 48). TGF- β 1 as a key fibrogenic mediator can enhance extracellular matrix deposition and inhibit collagenase activity (25, 49, 50). In conjunction with the decrease in α -SMA expression by oltipraz in activated stellate cells, oltipraz suppressed the increased expression of TGF- β 1 during the activation of stellate cells. This was in parallel with the inhibition of procollagen expression. The altered gene expression by oltipraz in activated stellate cells strongly supported the notion that oltipraz inactivates activated stellate cells.

In the present study, the mechanistic basis for the inhibition of TGF- β 1 was further studied. Confocal microscopy revealed that oltipraz caused C/EBP β to translocate into the nucleus of stellate cells. The response element of C/EBP is present in the 5'-flanking region of the *TGF- β 1* gene. Therefore, we next assessed the role of C/EBP β in the suppression of TGF- β 1 gene expression. The reversal of TGF- β 1 gene expression by C/EBP-specific decoy ODN supported the essential role of C/EBP β as a molecular target for the suppression of TGF- β 1 by oltipraz in stellate cells. Thus, inhibition of extracellular matrix accumulation by oltipraz in the liver of cirrhotic rats may result from C/EBP β -mediated TGF- β 1 suppression in stellate cells (Fig. 6). Also, oltipraz inhibited TGF- β 3-inducible *TGF- β 1* reporter gene expression in cells transfected with pGL3-453, a TGF- β 1 promoter vector containing C/EBP-response element (S.G. Kim and M.K. Cho, unpublished data), which raised the possibility that C/EBP forms a negative protein complex. TGF- β 1 stimulates collagen synthesis in stellate cells, and Smad proteins are transcriptional activators for the collagen genes inducible by TGF- β 1 superfamily members (51, 52). Activation of C/EBP negatively regulates the expression of type I collagen gene (53, 54). Also, Smad proteins suppress C/EBP β -mediated transcriptional activation (55). Hence, activation of C/EBP β by oltipraz may antagonize Smad-mediated induction of collagen and activate collagenase for the resolution of fibers accumulated in the liver. Inhibition of TGF- β 1 expression in stellate cells via activation of C/EBP provides a pharmacological target for the treatment of LC.

Oltipraz is structurally related to the 1,2-dithiolthiones naturally found in cruciferous vegetables. Oltipraz significantly decreased tumor incidence and multiplicity in liver, colon, skin, and lung (14, 17, 56, 57). In particular, the drug inhibits aflatoxin B1-induced hepatocarcinogenesis (14, 17). Oltipraz inhibits hepatitis B virus transcription, which causes the elevation of p53 protein (58). In addition, oltipraz inhibits human immunodeficiency virus replication (59). Inhibition of hepatitis B virus replication and its chemopreventive efficacy by oltipraz may be of great value for the treatment of chronic liver diseases. One-year chronic toxicity studies in rats and dogs showed that the safe dosage level of oltipraz estimated was approximately 10–30 mg/kg/day (60). Oltipraz was originally used as a potential schistosomicidal agent in humans at the daily dose of 30 mg/kg (61). In the present study, oltipraz at the daily equivalent dose of 13 mg/kg or less was highly efficacious as a treatment for LC. Oltipraz is the first therapeutic agent that regenerates cirrhotic liver. The clinical use of oltipraz will fill an important need for patients with LC.

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Table 1**Representative parameters for liver function^a**

Treatment	ALT (unit/l)	AST (unit/l)	Bilirubin (mg/dl)	Cholesterol (mg/dl)
Untreated control (n=8)	55 ± 4	141 ± 18	1.06 ± 0.10	97 ± 6
Cirrhotic rats				
Vehicle (n=9)	138 ± 14*	275 ± 36*	2.74 ± 0.84	152 ± 30
Oltipraz 15 mg/kg (n=1)	124 ± 47	206 ± 24	1.75 ± 0.35	116 ± 9
Oltipraz 30 mg/kg (n=1)	110 ± 39	185 ± 22 [#]	1.37 ± 0.15	104 ± 7

^aLiver cirrhosis was induced as described in Materials and Methods. The cirrhotic rats were orally treated with oltipraz at the indicated doses (three times per week for 4 wk). The aminotransferase activities, total bilirubin, and total cholesterol contents in the plasma of cirrhotic rats were assayed using an automatic blood chemistry analyzer. The values represent the mean ±SE. Paired Student's *t* test was used to compare two group means (significant compared with untreated control, **P*<0.01; significant compared with cirrhotic rats treated with vehicle, [#]*P*<0.05).

Fig. 1

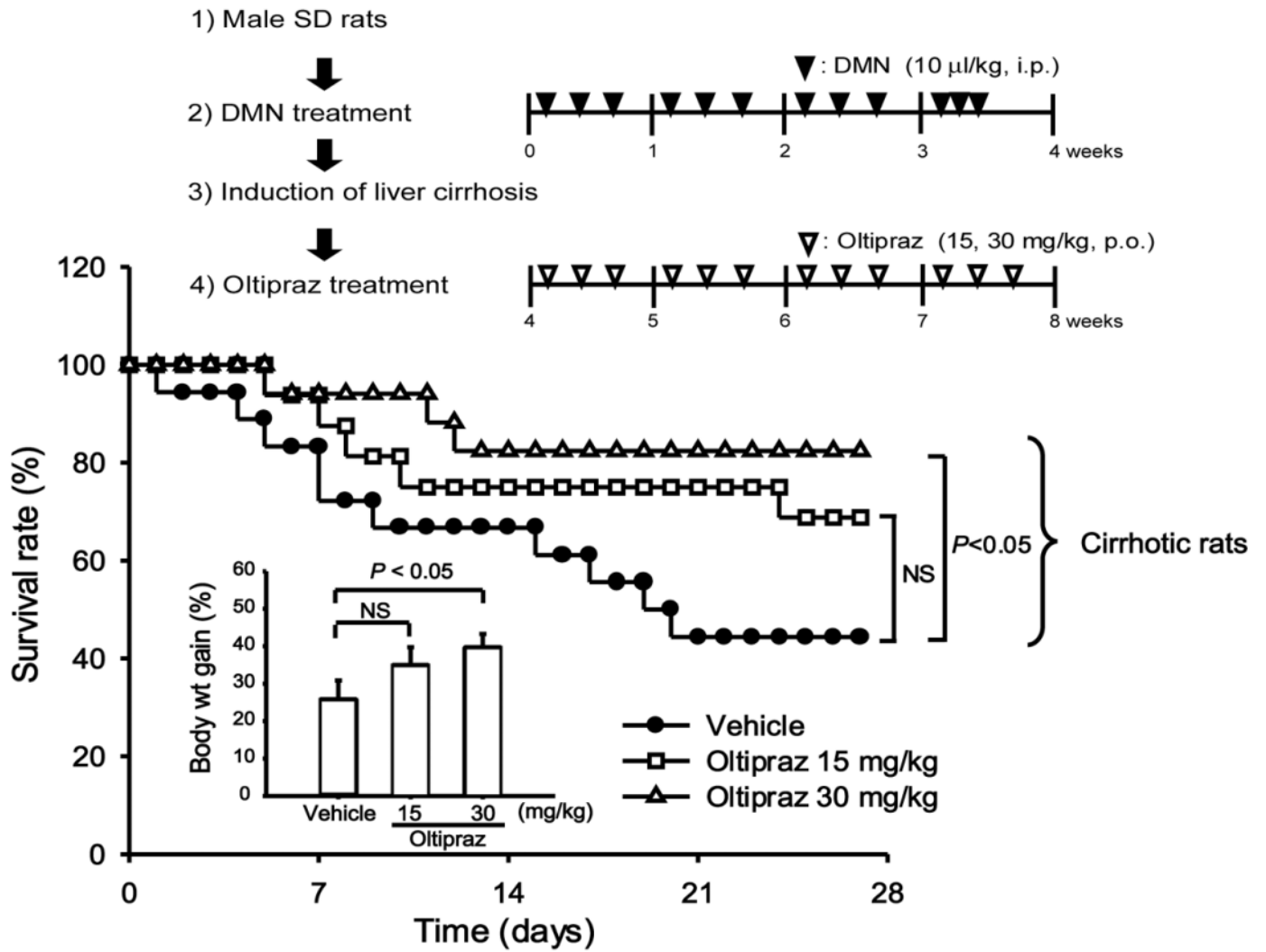


Figure 1. The survival rates of cirrhotic rats. Cirrhotic rats ($n=18$ per treatment) were orally treated with vehicle or oltipraz at a dose of 15 or 30 mg/kg three times per week for 4 wk. Survival rates were monitored daily during 4 wk of treatment regimen and were statistically analyzed on day 28 using χ -square test. Inset shows the body weight gain of cirrhotic rats alive. NS, not significant.

Fig. 2

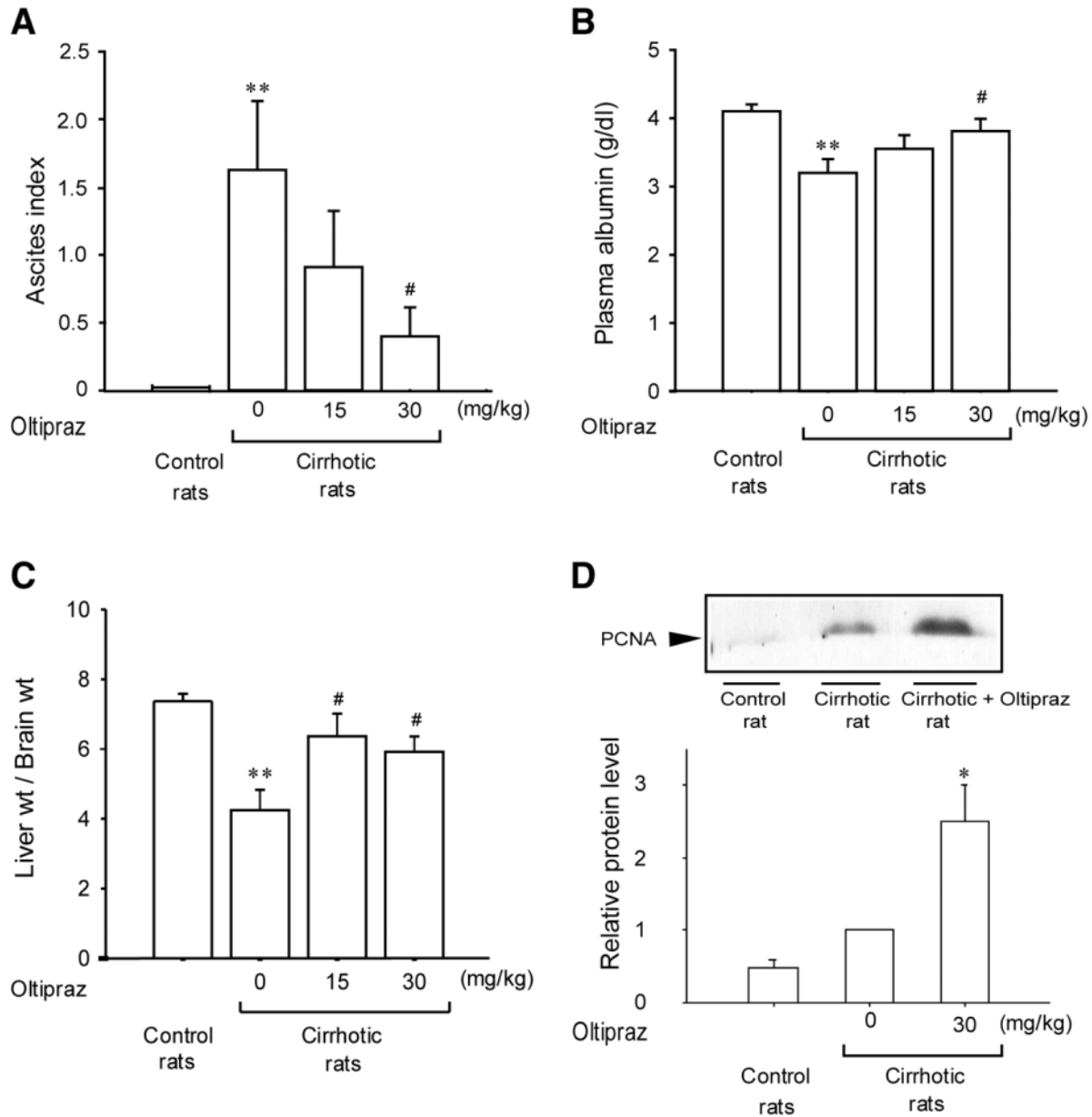


Figure 2. Improvement of liver function and regeneration of cirrhotic liver. A) Indexes of ascites. The ascites index was determined in cirrhotic rats treated with vehicle or oltipraz. The extent of ascites formation was graded as 0, no peritoneal fluid; 1, mild increase in peritoneal fluid (peritoneum was partially filled with fluid); 2, moderate increase (peritoneum was fully filled with ascites, which overflowed with mid-abdominal incision); 3, severe increase (abdominal girth was expanded by overproduction of ascites, which spurted out upon mid-abdominal incision) (significant compared with control, $**P<0.01$; significant compared with cirrhotic rats, $^{\#}P<0.05$; $n=8-11$). **B)** Plasma albumin contents. Plasma albumin levels were monitored in cirrhotic animals with or without oltipraz treatment (significant compared with control, $**P<0.01$; significant compared with cirrhotic rats, $^{\#}P<0.05$; $n=8-11$). **C)** Effect of oltipraz on the liver weight in cirrhotic rats. The liver-to-brain weight ratio was determined in cirrhotic rats treated with vehicle or oltipraz (significant compared with control, $**P<0.01$; significant compared with cirrhotic rats, $^{\#}P<0.05$; $n=8-10$). **D)** Western blot analysis of proliferating cell nuclear antigen (PCNA). PCNA was immunochemically stained in the hepatic nuclear proteins prepared from control rats or cirrhotic rats with or without oltipraz treatment. Each lane was loaded with 30 μg of protein. The relative level of PCNA was assessed by scanning densitometry (significant compared with cirrhotic rats, $**P<0.01$; $n=4$) (cirrhotic rats, 1).

Fig. 4

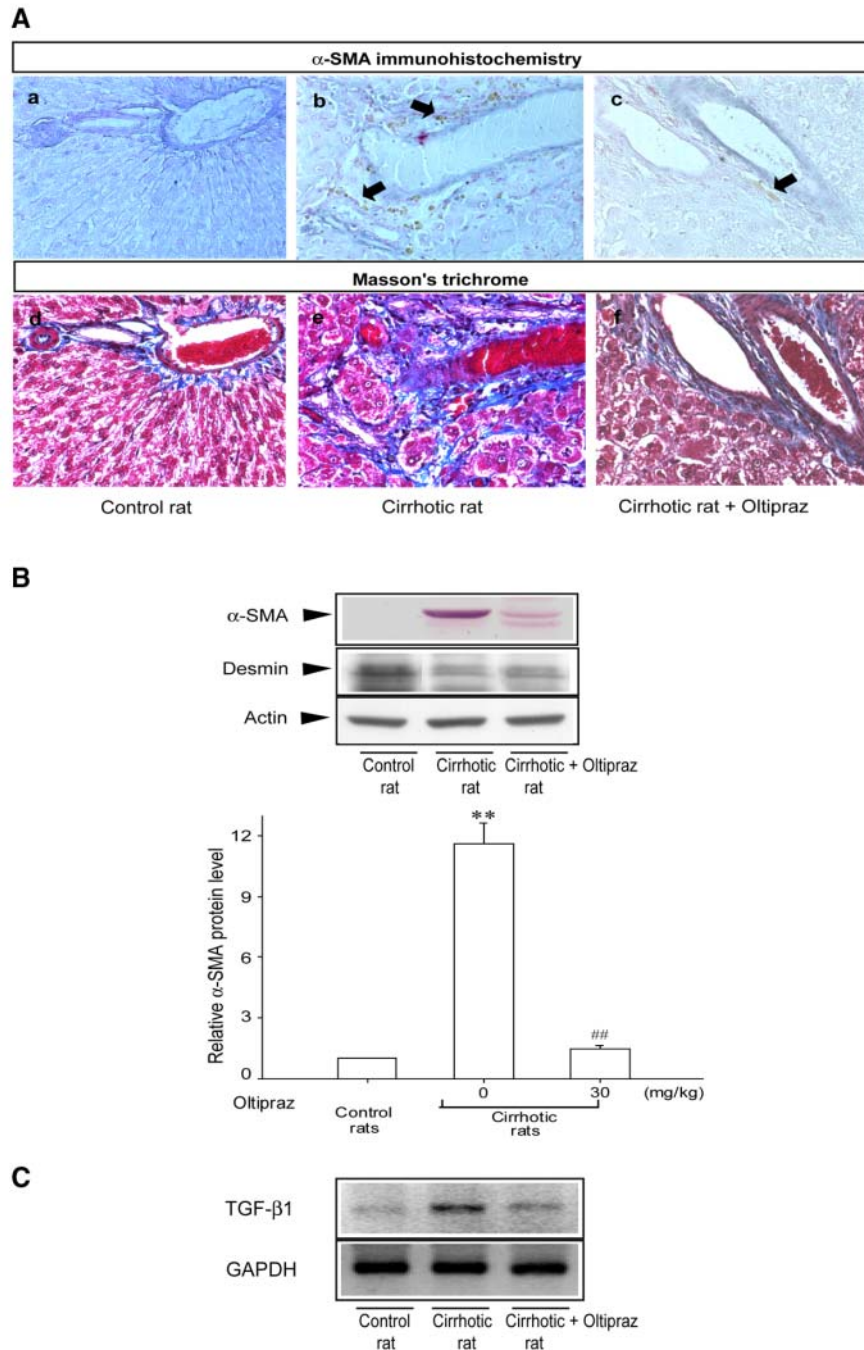


Figure 4. Inactivation of α -SMA-positive cells in the cirrhotic liver by oltipraz. A) Immunohistochemistry of α -SMA-positive cells in cirrhotic liver. The liver sections of healthy control rats (a) or cirrhotic rats treated with vehicle (b) or with 30 mg/kg oltipraz (c) were immunohistochemically stained with anti- α -SMA antibody (400 \times). Arrows indicate α -SMA-positive cells. Replicate samples were stained with Masson's trichrome to visualize accumulated fibers. **B)** Western blot analyses of α -SMA and desmin. α -SMA and desmin were immunochemically stained in the liver homogenates of control rats or cirrhotic rats treated with vehicle or 30 mg/kg oltipraz. Equal loading of protein was verified by actin immunoblot. Each lane was loaded with 30 μ g of protein. The relative levels of α -SMA were assessed by scanning densitometry (significant compared with control, $**P<0.01$; significant compared with cirrhotic rats, $###P<0.01$; $n=3$)(control level, 1). **C)** Inhibition of TGF- β 1 expression by oltipraz in cirrhotic liver. The level of TGF- β 1 mRNA in control rats or cirrhotic rats treated with vehicle or 30 mg/kg oltipraz was assessed by RT-PCR. The GAPDH mRNA was amplified as control. Results were confirmed by repeated experiments.

Fig. 5

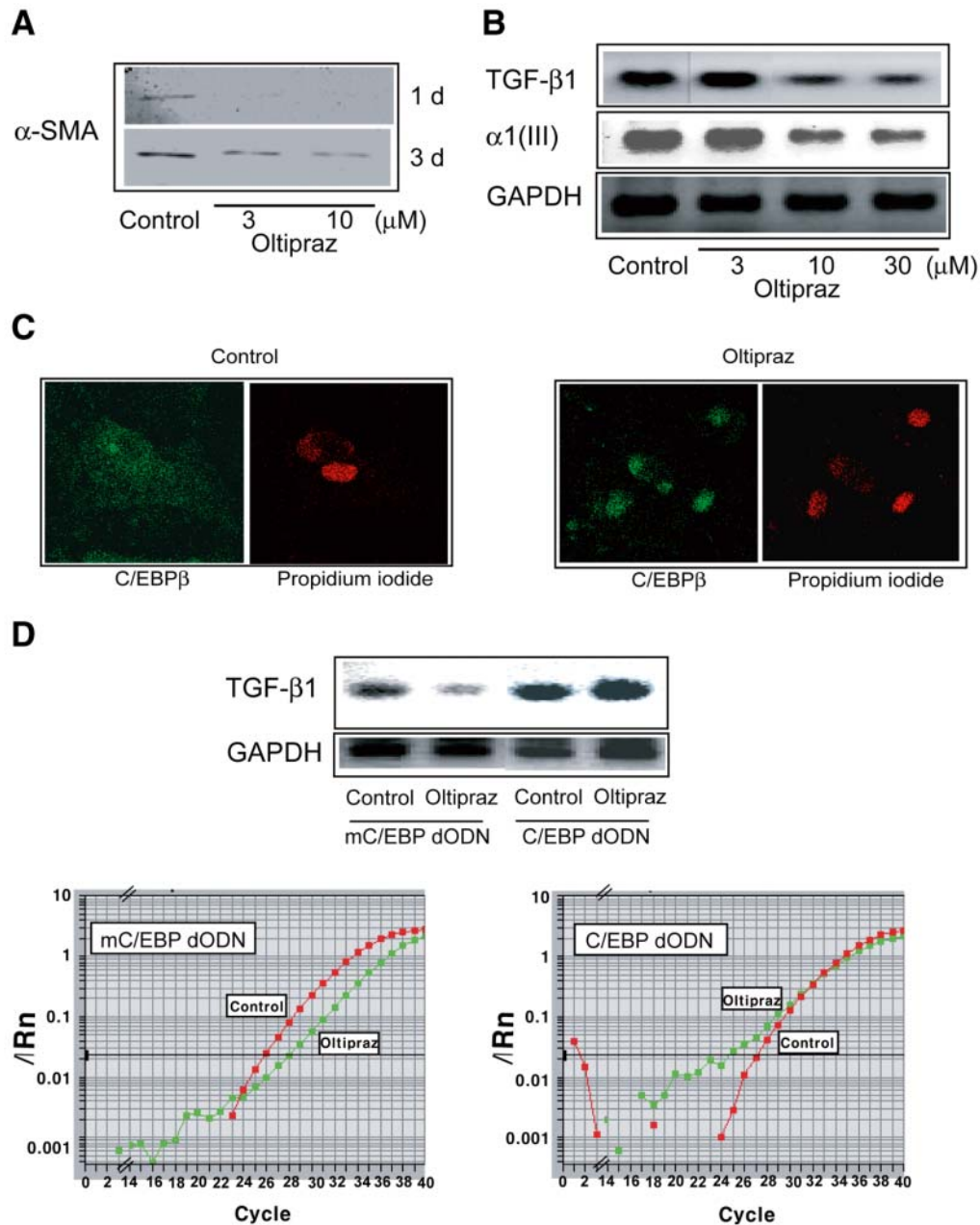


Figure 5. Inactivation of stellate cells by oltipraz. **A)** Inhibition of α -SMA expression by oltipraz in activated stellate cells. The level of α -SMA protein was measured in the lysates of stellate cells treated with 3–10 μ M oltipraz for 1 day or 3 days. **B)** Inhibition of TGF- β 1 and procollagen α 1(III) [α 1(III)] expression by oltipraz in activated stellate cells. Total RNA prepared from stellate cells treated with oltipraz (3–30 μ M) for 12 h was used for cDNA synthesis, and TGF- β 1 or procollagen α 1(III) mRNA expression was assessed by RT-PCR. The GAPDH mRNA was amplified by RT-PCR as control. **C)** Immunocytochemistry of C/EBP β in activated stellate cells. Nuclear translocation of cytosolic C/EBP β was monitored in stellate cells incubated with vehicle (dimethyl sulfoxide) or 30 μ M oltipraz for 1 h. The same fields were counterstained with propidium iodide. **D)** The role of C/EBP in the suppression of TGF- β 1 expression by oltipraz. Activated stellate cells were preincubated with 10 μ M of mutant C/EBP (mC/EBP) dODN or C/EBP dODN for 1 h and further incubated with or without 30 μ M oltipraz for 12 h. The levels of TGF- β 1 and GAPDH mRNA were assessed by RT-PCR analysis (upper). SYBR green real-time RT-PCR analysis was used to confirm the changes in the TGF- β 1 mRNA in stellate cells (lower). The amplification curve was right-shifted by the presence of oltipraz with the increase of C_T value in cells exposed to mutant C/EBP dODN. In contrast, oltipraz failed to increase C_T value in cells pretreated with C/EBP dODN. Results were confirmed by repeated experiments.

Fig. 6

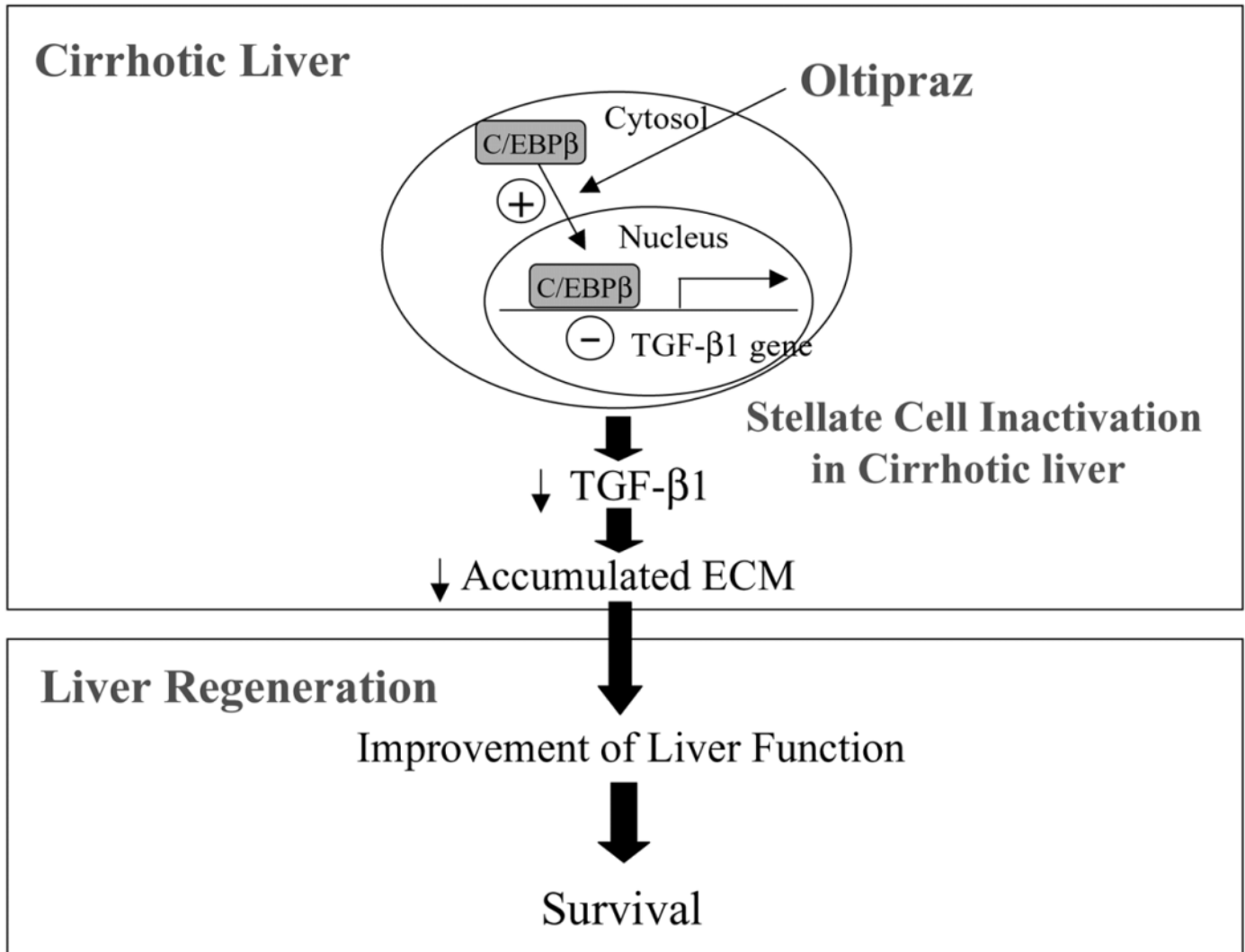


Figure 6. Schematic diagram illustrating the possible mechanism by which oltipraz regenerates cirrhotic liver. The activation of CCAAT/enhancer binding protein (C/EBP) by oltipraz inhibits transforming growth factor (TGF)- β 1 gene expression in stellate cells, which provides a molecular target for pharmacological treatment of liver cirrhosis. Oltipraz within the clinical dose range regenerates cirrhotic liver in the established cirrhotic rats as a result of inactivation of stellate cells, thereby reducing the intensities of cirrhotic nodules and improving the survival rate.