Regular Article

The Enhancing Effects of *S*-Allylcysteine on Liver Regeneration Are Associated with Increased Expression of mRNAs Encoding IGF-1 and Its Receptor in Two-Thirds Partially Hepatectomized Rats

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Two-thirds partial hepatectomy (PHx) was performed in rats, and the differences in effects between *S*-allylcysteine (SAC) and other sulfur-containing compounds on regeneration of the remaining liver and restoration of the injury were examined. Three days after two-thirds PHx, rats treated with 300 mg/kg/d, *per os* (*p.o.*) SAC showed a 1.2-fold increase in liver weight per 100 g body weight compared with saline-treated controls. In contrast, *S*-methylcysteine (SMC) (300 mg/kg/d, *p.o.*) or cysteine (Cys) (300 mg/kg/d, *p.o.*) did not have a regeneration-promoting effect. In the comparison with control rats, the regenerating liver of SAC-treated rats showed a significantly higher 5-bromo-2′-deoxyuridine labeling index on day 1. In contrast, serum alanine aminotransferase activity, which increases following PHx, was significantly inhibited by SAC and SMC (but not Cys) on day 1 after two-thirds PHx. In addition, SAC induced increases in insulin-like growth factor (IGF)-1 and its receptor mRNA expressions at 1 h after two-thirds PHx, and it increased phosphorylation of extracellular signal-regulated kinase (ERK)2 and Akt at 3 h after two-thirds PHx without affecting serum growth hormone levels. These results demonstrate that SAC is a mitogenic effector of normal remnant liver and promotes recuperation of liver function after two-thirds PHx. Moreover, SAC-induced proliferative effects are mediated *via* increased mRNA expressions of IGF-1 and its receptor and subsequent phosphorylation of ERK2 and Akt.

Key words partial hepatectomy (PHx); liver regeneration; *S*-allylcysteine (SAC); insulin-like growth factor (IGF)-1; serum alanine aminotransferase (ALT)

INTRODUCTION

The liver exhibits the unique physiologic capability of regeneration.[1)] Liver regeneration is usually quiescent, with no cell proliferation occurring. However, after extensive hepatic resection, such as resulting from partial hepatectomy (PHx), the remnant liver proliferates to reinstate the original mass within about 2 weeks.[1,2)] During liver regeneration, various growth factors (*e.g.*, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, and transforming growth factor (TGF)-α) are produced and stimulate the proliferation of hepatocytes.[1–4)]

The sulfur-containing compound, *S*-allylcysteine (SAC), is found in aged garlic extract and can be obtained following 20-month incubation of fresh garlic in ethanol.[5)] During the extraction process, concentrations of sulfur-containing compounds such as SAC, *S*-methylcysteine (SMC), and *S*-1-propenycysteine increase markedly.[6,7)] The study of SAC has included many reports of its antioxidant activities, such as scavenging of reactive oxygen species or activation of nuclear factor-E2-related factor 2 (Nrf2) factor.[6,8,9)] Due to its antioxidant effects, SAC reduces liver toxicity caused by acetaminophen or carbon tetrachloride.[10,11)] SAC also exhibits neuroprotective effects against Alzheimer's disease and chemopreventive effects against *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced gastric carcinogenesis.[12,13)] However, little information has been accumulated regarding SAC-induced proliferation of various cell types. Nam *et al.* reported that, in mice, SAC enhances division and differentiation of neuroblasts due to increased expression of serotonin 1A receptors in the dentate gyrus.[14)] We hypothesized that SAC increases the expression levels of proliferation-associated factors in partially hepatectomized rats.

Thus, the effect of SAC compared to cysteine (Cys) and SMC (Fig. 1), which also contain sulfur, on regeneration of the remaining liver and restoration of the injury in rats that underwent two-thirds PHx was examined.

Serum concentrations of liver-specific cytosolic transaminases (*e.g.*, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) were measured to determine whether SAC protects liver integrity after two-thirds PHx. Furthermore, to examine differences in gene expressions related to liver regeneration in response to SAC injections, the mRNA expressions of various growth factors and associated receptors (*e.g.*, HGF, IGF-1, and TGF-α) were measured using TaqMan real-time RT-PCR analysis.

In addition, to examine the involvement of p44/42 mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase (ERK)1/2) and Akt in the mitogenic pathway induced by SAC, their phosphorylation activities were measured by Western blotting.

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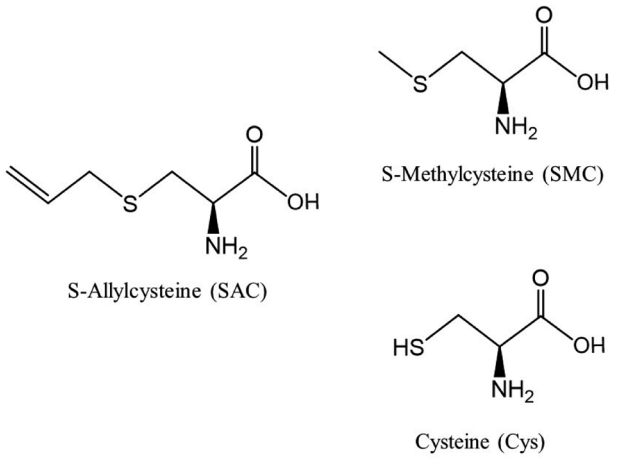

Fig. 1. Structural Formulas of SAC and Sulfur-Containing Compounds

MATERIALS AND METHODS

Handling of Animals Male Wistar rats (6–7 weeks old; body weight 170–200 g) were obtained from Sankyo Labo Service Corp. (Tokyo, Japan). The experimental animal-breeding facility was controlled to a room temperature of 18–26°C. The feeding/water supply to the rats was available *ad libitum*. All rats used in this study were handled humanely in accordance with the Guidelines for the Care and Use of Laboratory Animals of Josai University.

Two-Thirds PHx and Drug Administration Two-thirds PHx was performed in the rats based on the method of Higgins and Anderson.[15] In brief, under isoflurane anesthesia, the lateral attachment of the diaphragm and the left lateral hepatic lobes were ligated equivalent to approximately two-thirds of the total liver weight and removed. In the sham operation, a brief operation was performed on the peritoneal cavity, and it was then closed, but with neither ligation nor excision.

The rats were randomly divided into four groups that received oral administration of vehicle (saline, 0.5 mL/kg), SAC (300 mg/kg), SMC (300 mg/kg), or Cys (300 mg/kg, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) once per day (at 10:00). Administration of these drugs was performed daily for 1–12 d unless otherwise indicated.

5-Bromo-2′-deoxyuridine (BrdU) Immunohistochemistry To evaluate the hepatocyte mitotic index of remnant liver after two-thirds PHx, hepatocyte DNA synthesis activity was examined using a BrdU immunohistochemical technique based on a previously described method.[16,17] The thymidine analogue, BrdU, becomes part of the newly synthesized DNA in actively dividing cells. BrdU (50 mg/kg, intraperitoneally (i.p.)) was administered to each rat that was anesthetized with isoflurane 2 h later, at which time the liver was removed. In brief, the removed liver was fixed in Lillie's buffered formalin (10% formalin, 0.44% sodium dihydrogenphosphate dihydrate, 2.58% disodium hydrogenphosphate 12-water) and embedded in paraffin. To detect BrdU, liver sections cut with a microtome were placed in 2 mol/L HCl for 30 min for denaturation and then rinsed twice in phosphate-buffered saline. Next, liver sections were incubated with a biotinylated monoclonal antibody against BrdU (BrdU Immunohistochemistry Assay kit, Exalpha Biologicals, Shirley, MA, U.S.A.), followed by detection with a streptavidin-horseradish peroxidase conjugate (Exalpha Biologicals). Hematoxylin was used as a counterstain. The percent of BrdU-positive nuclei per 1000 nuclei was calculated in random fields viewed with a light microscope and considered the labeling index.

Table 1. TaqMan RT-PCR Primers and Probes

Gene	Accession number	Assay ID
HGF	NM_017017	Rn00566673_m1
Met	NM_031517	Rn00580462_m1
IGF-1	NM_001082477	Rn00710306_m1
IGF-1 receptor	NM_052807	Rn00583837_m1
TGF-α	NM_031199	Rn00446234_m1
TGF-α receptor	NM_031507	Rn01434447_m1
GAPDH	NM_017008	Rn01775763_g1

Measurement of Serum Transaminases and Growth Hormone Levels To determine whether SAC, SMC, or Cys affected liver function in rats subjected to two-thirds PHx, serum concentrations of transaminases (ALT and AST) were measured. The serum was obtained by centrifuging the collected venous blood sample. ALT and AST activities of obtained serum samples were assayed using a Transaminase CII-Test Wako kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), as directed by the manufacturer. The enzyme reaction of this assay was initiated by substrate to 4-aminoantipyrine. One international unit (IU) is defined as enzyme activity to change a substrate of 1 μmol per one minute at 25 °C.

Serum growth hormone (GH) levels were assayed by the Rat GH enzyme-linked immunosorbent assay (ELISA) kit (FUJIFILM Wako Shibayagi Corp., Gunma, Japan), according to the manufacturer's instructions.

RNA Extracted and Quantitative RT-PCR To quantitatively examine expressions of various genes in the liver of animals subjected to two-thirds PHx, quantitative RT-PCR was performed based on the TaqMan assay. TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) was used for isolation of total RNA from liver. Briefly, liver was homogenized in TRIzol reagent (1 mL/100 mg Liver), 200 μL of chloroform were added, samples were centrifuged, and then the aqueous phase was removed to a new tube. Next, RNA was precipitated by isopropyl alcohol from the obtained aqueous phase. The RNA was rinsed with 75% ethanol.

Total RNA was reverse transcribed to cDNA with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, U.S.A.), as specified by the manufacturer. The cDNA was then used for quantitative RT-PCR with the StepOnePlus Real-Time PCR System and TaqMan Fast Advanced Master Mix (Applied Biosystems), as specified by the manufacturer. Predesigned TaqMan probes and primers for the following target genes were used: HGF, Met (HGF receptor), IGF-1, IGF-1 receptor, TGF-α, TGF-α receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). The GAPDH mRNA level present in each sample was used to normalize data for each sample. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA levels.[18]

Measurement of Phosphorylated p44/42 MAPK (ERK1/2) and Akt To examine phosphorylation of p44/42 MAPK (ERK1/2) and Akt in the liver of animals subjected to two-thirds PHx, their phosphorylation was identified by Western blotting. TRIzol reagent (Invitrogen) was used for isolation of protein from liver. Briefly, liver was homogenized

in TRIzol reagent (1 mL/100 mg Liver), 200 μL of chloroform were added, samples were centrifuged, and then the phenol-chloroform phase was removed to a new tube. Next, protein was precipitated by isopropyl alcohol from the obtained protein sample and rinsed with 95% ethanol containing 0.3 M guanidine hydrochloride.

An immunoblot assay was performed based on the previously described method.[19] In brief, the protein samples redissolved in 1% sodium dodecyl sulfate (SDS) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-phospho-p44/42 ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) or anti-phospho-p60 Akt antibody (Cell Signaling Technology). Blots were developed by enhanced chemiluminescence following incubation with a secondary antibody conjugated with horseradish peroxidase. The total-ERK1/2 or Akt antibody present in each sample was used to normalize data for each phosphorylation activity (Cell Signaling Technology).

Statistical Analysis Data are shown as means ± standard error of the mean (S.E.M.). ANOVA was used for group comparisons of unpaired data followed by Dunnett's multiple comparison tests for *post hoc* analysis. Statistical significance was defined as $p < 0.05$.

RESULTS

Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds after Two-Thirds PHx To evaluate the proliferative effects of SAC and other sulfur-containing compounds on liver regeneration, SAC (300 mg/kg) was administered orally to the partially hepatectomized rats, and its effects were compared with the effects of SMC (300 mg/kg) or Cys (300 mg/kg). The evaluation of liver regeneration used the remnant liver weight per 100 g body weight (LW/100 gBW).[20] The LW/100 gBW of the sham operation group was 3.08 ± 0.09. After two-thirds PHx, the peak LW/100gBW in control (saline) rats was observed at day 5, whereas it peaked at day 3 in SAC-treated rats (Fig. 2-I). The LW/100 gBW at day 3 after two-thirds PHx was about 1.2-fold higher in SAC-treated rats than in saline-treated control rats. The second peak in the ratio of LW/100 gBW was observed at day 10 after two-thirds PHx, with liver mass almost restored to pre-PHx levels. There was no significant difference in liver weight from days 1 through 12 between rats treated with SAC and controls other than at days 3 and 5. In contrast, from days 1 through 12, neither SMC nor Cys induced a significant change in liver weight compared with saline-treated controls after two-thirds PHx (Fig. 2-I). On the other hand, there was no significant difference in body weight between treatment groups (Fig. 2-II).

Dose-Dependent Effects of SAC and Other Sulfur-Containing Compounds at Day 3 after Two-Thirds PHx As proliferative effects of SAC observed on day 3 in partially hepatectomized rats, the relationship between change in LW/100gBW at day 3 after two-thirds PHx and SAC dose was examined and compared with the relationships seen in SMC- and Cys-treated rats. As shown in Fig. 3-I, SAC (1–700 mg/kg) stimulated liver regeneration in a dose-dependent fashion by day 3 after two-thirds PHx. The maximum effect was found at an SAC dose of 300 mg/kg. In contrast, neither SMC (1–700 mg/kg) nor Cys (1–700 mg/kg) significantly increased

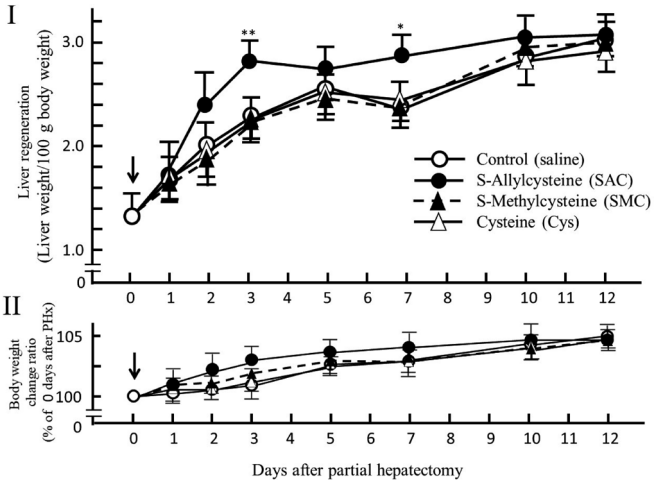

Fig. 2. Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds after Two-Thirds PHx

Method for preparing model rats, two-thirds partially hepatectomized rats, of liver regeneration, as described in Materials and Methods. (I) Liver weight /100 g body weight. (II) Body weight change rate. Control (○; saline 0.5 mL/kg, $n = 8$), SAC (●; 300 mg/kg, $n = 5$), SMC (▲; 300 mg/kg, $n = 5$), or Cys (△; 300 mg/kg, $n = 5$) was given orally once per day (at 10:00) on days 1 to 12 after PHx. The arrow shows the date of PHx and of starting drug treatment. Data are expressed as liver weight per 100 g body weight. Values are given as means ± S.E.M. $*p < 0.05$; $**p < 0.01$ compared with saline-treated controls.

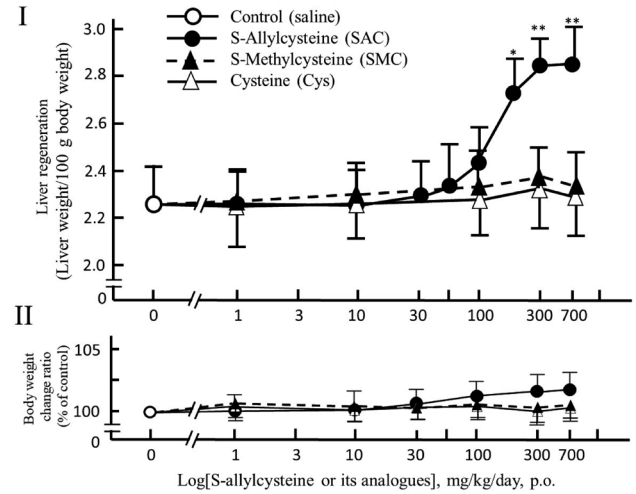

Fig. 3. Dose-Dependent Effects of SAC and Other Sulfur-Containing Compounds at Day 3 after Two-Thirds PHx

Method for preparing model rats, two-thirds partially hepatectomized rats, of liver regeneration, as described in Materials and Methods. (I) Liver weight /100 g body weight. (II) Body weight change rate. Control (○; saline 0.5 mL/kg, $n = 8$), SAC (●; 1–700 mg/kg, $n = 5$), SMC (▲; 1–700 mg/kg, $n = 5$), or Cys (▲; 1–700 mg/kg, $n = 5$) was given orally once per day (at 10:00) on day 3 after two-thirds PHx. Data are expressed as liver weight per 100 g body weight. Values represent means ± S.E.M. $*p < 0.05$; $**p < 0.01$ compared with saline-treated controls.

LW/100gBW at the doses tested (Fig. 3-I). On the other hand, there was no significant difference in body weight between treatment groups (Fig. 3-II).

Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds on Hepatocyte DNA Synthesis after Two-Thirds PHx The liver tissue image of Fig. 4 shows hepatocytes synthesizing DNA in the remnant liver of rats treated with saline (I), SAC (II), SMC (III), or Cys (IV) on day 1 after two-thirds PHx. Liver tissues were examined using BrdU immunochemical staining. Hepatocyte nuclei

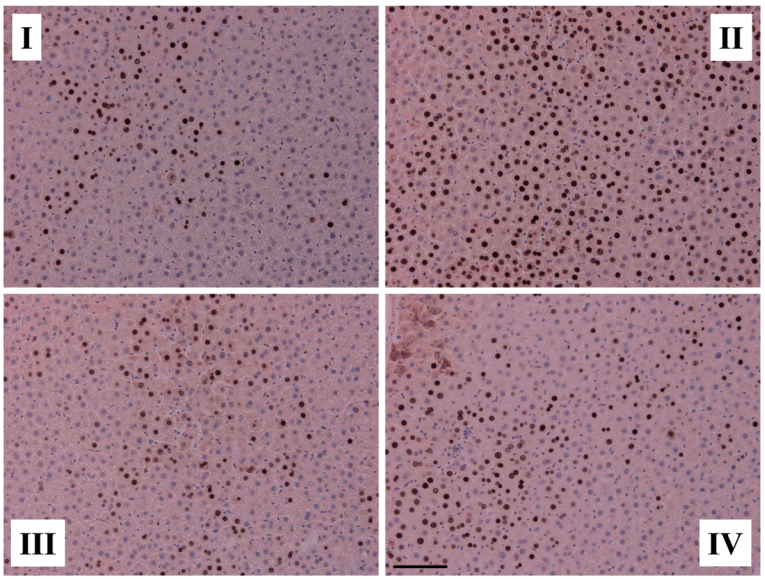

Fig. 4. Distribution of Hepatocytes Synthesizing DNA at Day 1 after Two-Thirds PHx

The liver tissue images show hepatocytes synthesizing DNA in the remnant liver of rats treated with saline, SAC, SMC, or Cys on day 1 after two-thirds PHx. Hepatocytes synthesizing DNA were determined by the BrdU immunohistochemical technique as described in Materials and Methods. Hepatocyte nuclei showing active DNA synthesis are stained dark brown. Hematoxylin was used as a counterstain. (I), Control (saline; 0.5 mL/kg, *per os* (*p.o.*)); (II), SAC (300 mg/kg, *p.o.*); (III), SMC (300 mg/kg, *p.o.*); (IV), Cys (300 mg/kg, *p.o.*). Bar, 20 μm.

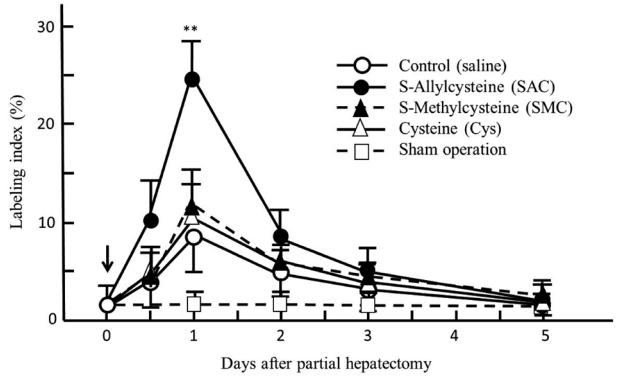

Fig. 5. Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds on the 5-Bromo-2′-deoxyuridine Labeling Index on Days 0–5 after Two-Thirds PHx

The ratio of BrdU incorporation into hepatocyte DNA is quantified by the labeling index. The BrdU labeling index in control- (○; saline 0.5 mL/kg, *p.o.*, $n=8$), SAC- (●; 300 mg/kg, *p.o.*, $n=5$), SMC- (▲; 300 mg/kg, *p.o.*, $n=5$), or Cys- (▲; 300 mg/kg, *p.o.*, $n=5$) treated rats determined as described in Materials and Methods. Open squares (□) represent sham-operated rats. The arrow shows the date of PHx and of starting drug treatment. Values are given as means ± S.E.M. $^{**}p<0.01$ compared with saline-treated controls.

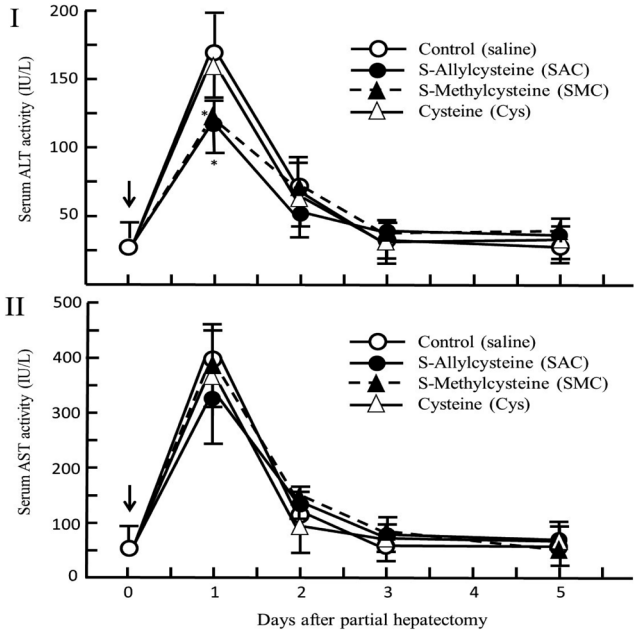

Fig. 6. Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds on the Serum Activities of Liver-Specific Cytosolic Transaminases (ALT, AST) after Two-Thirds PHx

Time-dependent effects of control (○; saline 0.5 mL/kg, $n=8$), SAC (●; 300 mg/kg, *p.o.*, $n=5$), SMC (▲; 300 mg/kg, *p.o.*, $n=5$), or Cys (▲; 300 mg/kg, *p.o.*, $n=5$) on the serum transaminase activities on days 0–5 after two-thirds PHx, as described in Materials and Methods. (I), serum ALT activity; (II), serum AST activity. The arrow shows the date of PHx and starting drug treatment. Values are given as means ± S.E.M. $^{*}p<0.05$ compared with saline-treated controls.

showing active DNA synthesis were stained dark brown. BrdU-positive cells were more frequent in SAC-treated rats than in SMC- or Cys-treated rats on day 1 after two-thirds PHx (Fig. 4).

Figure 5 shows the time-dependent effect of SAC on the BrdU labeling index (BrdU-LI) of hepatocytes on days 0 to 5 after two-thirds PHx. The BrdU-LI of hepatocytes was less than 0.2% in sham-operated rats. This index increased moderately and peaked on day 1 after surgery in saline-treated controls and then decreased to baseline by day 3. In contrast, the BrdU-LI in hepatocytes of rats treated with SAC increased about 2.8-fold on day 1 after two-thirds PHx compared to saline-treated control rats and returned to baseline by day 3. These results suggest that SAC significantly enhances the proliferative effects of hepatocytes compared with saline-treated controls after two-thirds PHx. The BrdU-LI values of rats treated with SMC and Cys were not significantly different from the saline-treated controls (Fig. 5).

Time-Dependent Effects of SAC and Other Sulfur-Containing Compounds on Serum Transaminase Levels We measured the activities of the serum transaminases (ALT

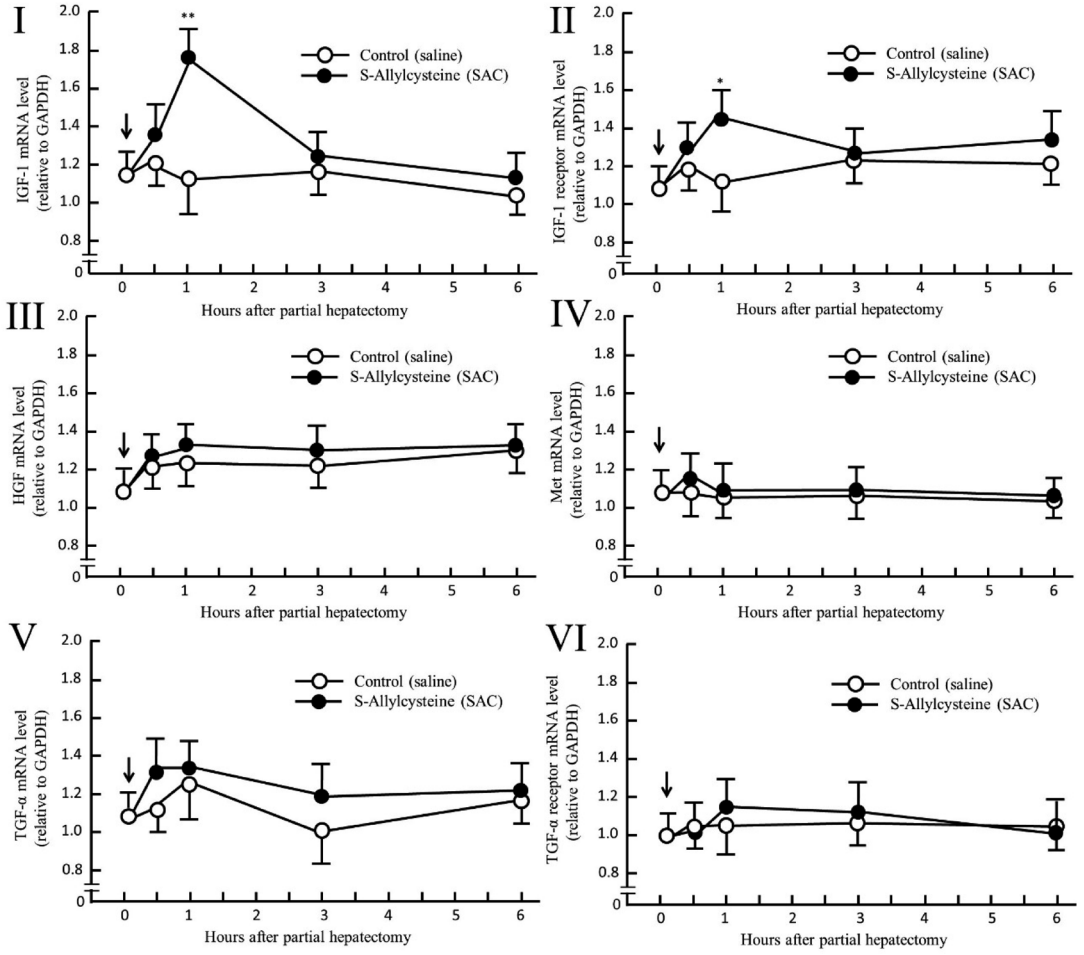

Fig. 7. Time-Dependent Effects of SAC on mRNA Expression Levels after Two-Thirds PHx

mRNA expression levels in rats subjected to two-thirds PHx were measured using RT-PCR based on the TaqMan assay, as described in the Methods. (I) IGF-1, (II) IGF-1 receptor, (III) HGF, (IV) Met (HGF receptor), (V) TGF-α, and (VI) TGF-α receptor. Control (○; saline 0.5 mL/kg, $n=8$) or SAC (●; 300 mg/kg $n=5$) was given orally once per day (at 10:00) at 1 to 6 h after PHx. Data for each sample were normalized to the GAPDH mRNA level in each sample. The arrow shows that date of PHx and of starting drug treatment. Values are given as means ± S.E.M. $*p<0.05$; $**p<0.01$ compared with saline-treated controls.

and AST) to determine whether SAC affected liver function after PHx. After two-thirds PHx in saline-treated control rats, the peak serum ALT activity was observed at day 1 (Fig. 6-I). On day 3 after PHx, serum ALT activity decreased rapidly up to pre-PHx levels, and this continued until day 5. In contrast, after two-thirds PHx, ALT activity in SAC- and SMC-treated rats at day 1 decreased significantly compared with control rats. In Cys-treated rats, there was no decrease compared with saline-treated controls in serum ALT activity (Fig. 6-I).

In saline-treated control rats after two-thirds PHx, serum AST activity increased rapidly and peaked on day 1 (Fig. 6-II). SAC, SMC, and Cys did not affect AST activity relative to saline-treated controls on days 1–5 (Fig. 6-II).

Effect of SAC on mRNA Expression Levels after Two-Thirds PHx We hypothesized that SAC-stimulated liver regeneration involves effects mediated by growth factors. Therefore, mRNA expression levels of genes encoding various growth factors (IGF-1, HGF, and TGF-α) and their receptors (IGF-1 receptor, Met; HGF receptor; TGF-α receptor) were measured using RT-PCR (Fig. 7). IGF-1 mRNA expression levels increased rapidly following SAC administration, peaking (at an about 1.6-fold increase compared with the saline-treated controls) at 1 h after PHx. Thereafter, IGF-1 mRNA expression levels decreased rapidly, reaching basal levels by 6 h after PHx (Fig. 7-I). IGF-1 receptor mRNA expression levels in SAC-treated rats showed a weaker response than IGF-1 mRNA, with an about 1.3-fold increase compared with saline-treated controls at 1 h after two-thirds PHx (Fig. 7-II).

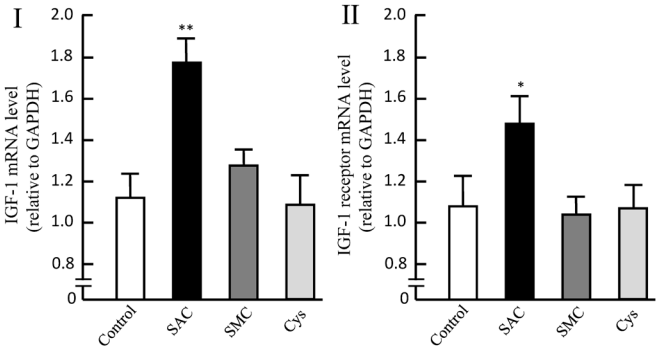

Fig. 8. Effects of SAC and Other Sulfur-Containing Compounds on mRNA Expression Levels at 1 h after Two-Thirds PHx

The mRNA expression levels in rats subjected to two-thirds PHx were measured using RT-PCR based on the TaqMan assay, as described in Materials and Methods. (I) IGF-1, (II) IGF-1 receptor. Control (saline 0.5 mL/kg, $n=8$), SAC (300 mg/kg, $n=5$), SMC (300 mg/kg, $n=5$), or Cys (300 mg/kg, $n=5$) was given orally once per day (at 10:00) at 1 h after two-thirds PHx. Data for each sample were normalized to the GAPDH mRNA level in each sample. Values are given as means ± S.E.M. $*p<0.05$; $**p<0.01$ compared with saline-treated controls.

In contrast, there was no significant change in HGF, TGF-α, HGF receptor, or TGF-α receptor mRNA in rats treated with SAC compared with saline-treated controls (Figs. 7-III–VI).

Whether treatment with SMC or Cys increased mRNA expression levels of IGF-1 and its receptor at 1 h after PHx was also examined (Fig. 8). Neither SMC nor Cys induced significant changes in IGF-1 or IGF-1 receptor mRNA levels at 1 h after PHx (Fig. 8).

Effect of SAC Administration on Serum GH Levels Next, whether SAC-induced increases in IGF-1 mRNA expression levels were mediated by elevating serum GH levels was examined. Therefore, serum GH concentrations were measured using a GH ELISA kit. As shown in Fig. 9, there was no significant difference in serum GH levels between rats treated with SAC compared with saline-treated controls within 3 h after two-thirds PHx (Fig. 9).

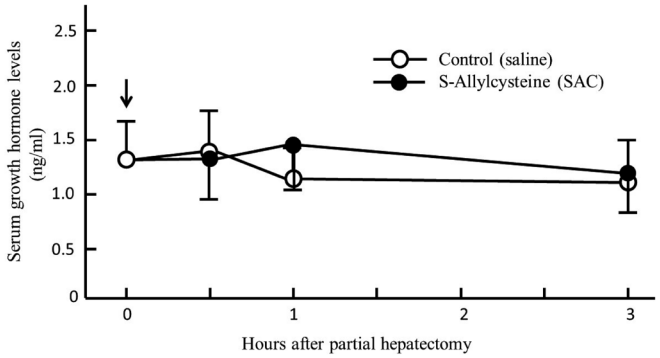

Fig. 9. Time-Dependent Effects of SAC on Serum Growth Hormone Levels after Two-Thirds PHx

Serum growth hormone levels in rats subjected to two-thirds PHx were measured using a Rat GH ELISA kit, as described in Materials and Methods. Control (○: saline 0.5 mL/kg, $n = 5$) or SAC (●: 300 mg/kg, $n = 5$) was given orally once per day (at 10:00) at 1 to 3 h after PHx. The arrow shows the date of PHx and of starting drug treatment. Values are given as means ± S.E.M.

Effect of SAC on Phosphorylation of ERK1/2 and Akt after Two-Thirds PHx Next, phosphorylation activities of ERK (p44 ERK1, p42 ERK2) and Akt were measured to determine whether IGF-1 mRNA induced by SAC administration stimulated an intracellular signal transduction factor. After two-thirds PHx, phosphorylated p42 ERK2 of control rats in the remnant liver was increased at 1 h, and then this phosphorylation activity decreased until pre-PH levels at 3 h. In contrast, phosphorylated p42 ERK2 of SAC-treated rats lasted until 3 h after PHx (Fig. 10-I). On the other hand, p44 ERK1 phosphorylation was not affected by PHx and SAC treatment (Fig. 10-I).

The phosphorylation pattern of Akt was similar to that of p42 ERK2 after two-thirds PHx. Akt phosphorylation activity was significantly increased in SAC-treated rats compared to saline-treated rats at 3 h after PHx (Fig. 10-II).

DISCUSSION

In this study, the effects of SAC on proliferation and regeneration of the remaining liver, restoration of liver function, and gene expression related to liver regeneration after PHx were examined.

LW/100 gBW increased significantly in SAC-treated rats at day 3 after two-thirds PHx compared with that in saline-treated controls (Figs. 2, 3). The liver weight of SAC-treated rats reached ≥90% of the pre-operative weight by day 3 after two-thirds PHx. SAC effects were dose-dependent and maximal at a dose of 300 mg/kg (Fig. 3). In previous reports, protective effects based on the antioxidant activity of SAC were generally observed at doses of 1 to 150 mg/kg.[21–24] Nam *et al.* reported that neuroblast proliferation caused by SAC in the mouse dentate gyrus was observed at a dose of 300 mg/kg.[14]

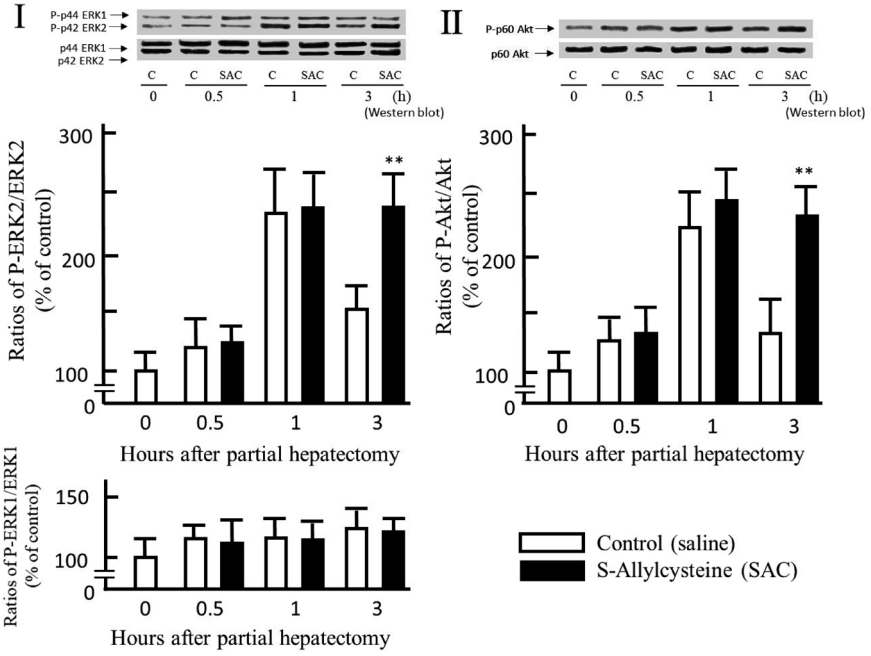

Fig. 10. Effects of SAC on Phosphorylation of ERK1/2 and Akt after Two-Thirds PHx

Phosphorylation of ERK1/2 and Akt was determined by Western blot analysis as described in Materials and Methods. (I) Phosphorylated ERK1/2 (P-p44 ERK1, P-p42 ERK2) and total ERK1/2 protein (p44 ERK1, p42 ERK2). (II) Phosphorylated Akt (P-p60 Akt) and total Akt protein (p60 Akt). Control (saline 0.5 mL/kg, $n = 5$) and SAC (300 mg/kg, $n = 5$) was given orally once per day (at 10:00) at 0.5 to 3 h after PHx. Data for each sample were normalized to the total antibodies in each sample. Values are given as means ± S.E.M. $^{**}p < 0.01$ compared with saline-treated controls.

The cell proliferative effect caused by SAC may thus occur at doses ≥200 mg/kg. These results indicate that administration of high-dose SAC accelerates remnant liver regeneration after two-thirds PHx.

SMC, which shows both antioxidant and anti-inflammatory effects,[25] did not accelerate remnant liver regeneration after two-thirds PHx in the present study. In previous studies, we demonstrated that antioxidant vitamins (*e.g.*, isoascorbic acid, vitamin E, and vitamin K) do not stimulate hepatocyte proliferation. In addition, anti-inflammatory drugs such as dexamethasone and ibuprofen do not accelerate remnant liver regeneration after two-thirds PHx.[19,26,27] These results suggest that SAC-induced acceleration of liver regeneration is unrelated to antioxidant or anti-inflammatory effects.

The liver is an organ containing much blood, and liver weight reflects not only the amount of liver tissue, but also blood volume. Therefore, the characteristic distribution of hepatocytes synthesizing DNA was also evaluated using BrdU immunochemical staining in remnant liver of SAC-treated rats (Figs. 4, 5). Liver regeneration after two-thirds PHx was also monitored using the mitotic index based on a previously established method, since this assay demonstrates nuclear BrdU incorporation into hepatocyte DNA.[16,17] The present results are consistent with a previous report[2] that showed that DNA synthesis in rat liver begins 12 h after two-thirds PHx and peaks at 24 h. The present data also showed that 300 mg/kg SAC induced a significant increase in the BrdU-LI on day 1 after two-thirds PHx relative to controls. We previously demonstrated the effects of glycyrrhizin and NIK-333, a vitamin A analogue, in accelerating liver regeneration after two-thirds PHx.[26,28] These compounds also induced peak hepatocyte-synthesizing DNA on day 1 after two-thirds PHx.

The serum activity of liver-specific cytosolic transaminases such as ALT and AST was evaluated *in vivo* after two-thirds PHx. In controls, ALT and AST activities increased quickly, peaked on day 1 after two-thirds PHx, and then returned to baseline by day 3 (Fig. 6). These transient increases may be in response to injury near the ligated zone of the liver. ALT activity was significantly lower in rats given SAC or SMC than in controls on day 1 after two-thirds PHx. Cys had no effect on the activity of either AST or ALT after two-thirds PHx. Previous reports have shown that SAC and SMC inhibit the biosynthesis of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and anti-inflammatory effects have been demonstrated in several knockout models, including rats with streptozotocin-nicotinamide-induced diabetes[29] or acetaminophen-induced hepatotoxicosis[10] and mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.[30] However, these cytokines are known to be liver regeneration-priming factors, and several knockout models did not demonstrate liver regeneration.[4,31] As shown in Figs. 2 and 3, neither SAC nor SMC was found to inhibit liver regeneration. In addition, previous studies have reported that glycyrrhizin, which accelerates liver regeneration and lowers serum transaminase activity after two-thirds PHx, inhibits the biosynthesis of IL-6 and TNF-α.[26,32] SAC and SMC may block prostaglandin synthesis, as well as production of other inflammatory mediators. However, why SAC and SMC did not decrease serum AST activity remains to be clarified. AST is present not only in the liver, but also in other tissues (*e.g.*, skeletal muscle), unlike ALT. SAC and SMC may decrease only ALT by a highly selective hepatoprotective effect.

SAC treatment increased IGF-1 and its receptor mRNA expressions at 1 h after two-thirds PHx (Figs. 7-I, -II) and phosphorylation of p42 ERK2 and Akt at 3 h after two-thirds PHx (Fig. 10). IGF-1 is one of the growth factors involved in cell proliferation and survival, and its intracellular signaling pathway includes the MAP kinase (p44 MAPK, ERK1, p42 MAPK, ERK2) pathway and the phosphoinositide 3-kinase (PI3K)–Akt pathway.[33] In a previous study, we reported that IGF-1 promoted hepatocyte proliferation by ERK2 *via* PI3K *in vitro*.[19,34] These results suggest that SAC-induced proliferative effects are mediated *via* increased expression of the mRNAs encoding IGF-1 and its receptor and activation of ERK 2 and Akt by secondary biosynthesized IGF-1. On the other hand, previous studies have demonstrated that p44 MAPK (ERK1) is not involved in hepatocyte proliferation *in vivo*.[35,36]

The biosynthesis of IGF-1 is stimulated by GH, which is secreted from the anterior pituitary gland.[37] GH promotes the expression of mRNA IGF-1 and its receptor through activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in hepatocytes.[38,39] It has been hypothesized that SAC increases IGF-1 by promoting GH secretion. However, serum GH levels in SAC-treated rats did not show a significant increase compared to saline-treated controls (Fig. 9). A possible explanation is that SAC may promote production of mRNAs for IGF-1 and its receptor by directly stimulating the GH receptor/JAK/STAT signaling pathway.

HGF, TGF-α, and their receptor mRNA levels in rats given SAC were not significantly higher than those of saline-treated controls, and no peaks in the expression of these mRNAs were observed within 6 h after two-thirds PHx (Figs. 7-III–VI). Michalopoulos and DeFrances reported that HGF and TGF-α mRNA expression levels peaked at 12 h after two-thirds PHx.[2] In addition, HGF is activated from the single-chain HGF (pro-HGF) inactive form by an increase in urokinase plasminogen activator immediately after two-thirds PHx, and plasma levels of HGF increase 10- to 20-fold within 1 h after PHx.[2]

Whether SMC or Cys treatment results in increases in the expressions of mRNAs encoding IGF-1 and its receptors at 1 h after two-thirds PHx was also examined (Fig. 8). Neither SMC nor Cys affected the production of IGF-1 or its receptor. The observed decrease in ALT activity in SMC-treated rats was thus not mediated by IGF-1 or its receptor.

SAC is a sulfate compound containing an allyl group in its structure (Fig. 1). Allyl compounds have stronger scavenger activity than non-allyl compounds such as SMC and *S*-propylcysteine.[6,40] In addition, it is also involved in Nrf2 activation. SAC activates Nrf2 by modifying cysteine residues in the Nrf2–keap1 complex.[6] An Nrf2 activation effect has also been reported with other allyl sulfate compounds, such as allyl isothiocyanate and allyl methyl trisulfide.[41,42] Therefore, the allyl group in SAC may play an important role in the bioactivity that results in increased expression of mRNA encoding IGF-1 and its receptor by directly stimulating specific signal-transducing proteins in the GH receptor/JAK/STAT pathway.

From a therapeutic perspective, the biological understanding of liver regeneration and techniques developed to facilitate the process have been used in living donor liver transplantation (LDLT), which is one of the choices for curing terminal-stage liver disease. However, this therapy imposes a heavy burden on the donor, with a transient postoperative decrease

in liver function, development of abdominal symptoms, and risks of complications such as bile leak or infection.[43–45] In addition, a long period (3–6 months) of medical treatment may be required before the donor can return to work or normal activities.[46] However, in the present study, SAC treatment accelerated liver regeneration in partially hepatectomized rats, suggesting that SAC administration could ease the physical and economic burdens on donors after LDLT.

In conclusion, it was established for the first time that SAC is a mitogenic effector of normal remnant liver and recuperation of liver function *in vivo* after two-thirds PHx. Moreover, SAC-induced proliferative effects were found to be associated with increased expression of mRNAs encoding IGF-1 and its receptor and subsequent phosphorylation of ERK2 and Akt.

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Conflict of Interest The authors declare no conflict of interest.

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