Antifibrogenic Effects of Magnesium Lithospermate B on Human Hepatic Stellate Cells and Thioacetamide-Induced Cirrhotic Rats

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Antifibrogenic Effects of Magnesium Lithospermate B on Human Hepatic Stellate Cells and Thioacetamide-Induced Cirrhotic Rats

Directed by Professor Kwang-Hyub Han

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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June 2008

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June 2008

ACKNOWLEDGEMENTS

I would like to express my special thanks to Prof. Kwang-Hyub Han who guided me in every step of this dissertation with warm and delicate attention. I am very grateful to Prof. Yong Han Paik, Prof. Hyun Chul Lee, Prof. Chanil Park and Prof. Kyungsoo Park who generously offered guidance and supports.

I am also deeply grateful to Sookin Chung, Sang Hun Lee, and So Hee Kang for their thoughtful supports in the experiments.

In addition, I really appreciate Dong Eun Lee in Department of Chemistry, Yonsei University who provided magnesium lithospermate B.

Author

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<ABSTRACT>

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Hepatic fibrosis refers to a wound-healing response to a variety of insults, ultimately leading to cirrhosis. Recently, magnesium lithospermate B (LAB), one of the major active components from *Salvia miltiorrhizae*, has been given attention for its anti-fibrogenic effects. The antifibrotic effect of LAB in an experimental rat model was demonstrated. The aim of this study was to elucidate the antifibrogenic mechanism of LAB. Sprague-Dawley rats were divided into 6 groups: the control group (n = 5), control + LAB group (n = 5), TAA 8-week group (n = 5), TAA + LAB 8-week group (n = 5), TAA 12-week group (n=5) and TAA + LAB 12-week group (n = 5). The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were tested. Masson's trichrome stain and α-smooth muscle actin (α-SMA) stain of liver sections were analyzed. The relative levels of mRNA expressions of α-SMA,

transforming growth factor-β (TGF-β) and type I collagen α1 were measured using real-time PCR. The human hepatic stellate cell (HSC) line was used for *in vitro* analysis. Cell cytotoxicity and proliferation were evaluated. Intracellular reactive oxygen species (ROS) formation was measured using H_2DCFDA labeling. Nuclear factor-κB (NF-κB) transcriptional activation induced by tumor necrosis factor-α (TNF-α) was measured using luciferase activity after virus vector transfer. Secreted type I collagen expression and phospho-extracellular signal regulated kinase (ERK) was evaluated using Western blots. LAB attenuated fibrosis in liver sections. The serum levels of AST and ALT were significantly lower in the TAA + LAB 8-week group than in the TAA 8-week group. LAB significantly decreased the mRNA levels of α-SMA, TGF-β and type I collagen α1. LAB treatment up to 40 μM did not show any discernable toxicity. Platelet-derived growth factor (PDGF)-induced proliferation of HSCs was suppressed by LAB. H_2O_2-induced ROS formation in HSCs was decreased by LAB. Western blot demonstrated that LAB decreased type I collagen secretion and phospho-ERK expression in HSCs. In conclusion, the results of this study suggest that LAB may attenuate the activation of human HSCs through antioxidant activity. LAB demonstrated an antifibrogenic effect on TAA-induced cirrhosis in Sprague-Dawley rats.

Key words: hepatic fibrosis, hepatic stellate cells, magnesium lithospermate B, antifibrotic agent

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I. INTRODUCTION

Hepatic fibrosis refers to a wound-healing response to a variety of insults, ultimately leading to cirrhosis.[1] The accumulation of the extracellular matrix (ECM) following liver injury is the main pathological alteration. The accumulation of ECM in the liver results from increased synthesis by activated hepatic stellate cells (HSC) and other fibrogenic cell types.[1] Numerous pharmaceutical agents have been tried to attenuate fibrosis.[2-5] However, these

agents have not been effective in clinical trials.[5]

Oxidative stress plays an important role in the initiation of hepatic fibrogenesis.[1] Increased reactive oxygen species (ROS) is directly fibrogenic and stimulates proliferation and invasiveness of HSC.[6] Mitogen-activated protein kinases (MAPKs) constitute a family of serine/threonine kinases that includes extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK.[7] The activation of these kinases occurs in response to a variety of stimuli in addition to oxidative stress.[8] Also, the nuclear factor-κB (NF-κB) signaling cascade has been implicated in liver injury from oxidative stress.[8] During cellular activation of HSCs, NF-κB activity is increased.[9]

Salvia miltiorrhizae is widely used as an herbal medicine in China for the treatment of vascular disease.[10] Magnesium lithospermate B (LAB) is one of the major active components from *Salvia miltiorrhizae*.[10] The renal protective effect of LAB has been reported.[11,12] Inhibition of ROS generation has been considered to be the major effect of LAB in renal protection.[12,13] The *in vivo* antifibrotic effect of LAB in an animal model has also been demonstrated.[14,15] However, the *in vitro* antifibrotic effect has not yet been clearly demonstrated. Furthermore, oxidative stress plays a major role in the thioacetamide (TAA)-induced rat cirrhosis model, and histological and biochemical changes are

similar to those observed in humans in this animal model.[16]

Therefore, the TAA-treated rat model was used to reveal the *in vivo* effect of LAB, and the *in vitro* effect of LAB was evaluated in the immortal human HSC cell line.

II. MATERIALS AND METHODS

1. Magnesium lithospermate B preparation

Slices of the dried roots of 1.0 kg of *Salviae miltiorrhizae* were placed in water at 100℃ for 2 hours. The heating water extract was dried through evaporation under reduced pressure at 70℃. The heating water extract was suspended in 500 mL of water, and the water extract was adjusted to pH 3.5 with hydrogen chloride (HCl) and extracted with saturated butyl alcohol (300 mL x 5 each). The butyl alcohol extract was evaporated to dryness under reduced pressure at 70℃. Then, the butyl alcohol extract was suspended in 300 mL of water and was washed with hexane (300 mL x 3 each). The water extract was evaporated to dryness under reduced pressure at 70℃. The water extract was extracted with ethyl acetate (300 mL x 3 each). The ethyl acetate extract and water extract were evaporated to dryness under reduced pressure at 70℃. The ethyl acetate extract and the water extract were purified by recrystallization

and fractional crystallization. The yield of the LAB as amorphous powder was 10 g from 1.0 kg of *Salviae miltiorrhizae*.

2. *In vivo* analysis

A. Animal preparation

All experimental procedures were performed according to the guidelines for the care and use of animals established by Yonsei University College of Medicine. Six-week-old male Sprague-Dawley rats were prepared. Hepatic fibrosis was induced by intraperitoneal injection of TAA (Sigma, St. Louis, MO, USA) at a dose of 200 mg/kg twice a week for 12 weeks. LAB at a dose of 40 mg/kg was fed with a gavage tube once daily for 12 weeks. Six experimental groups were studied: the control group (n = 5), control + LAB group (n = 5), TAA 8-week group (n = 5), TAA + LAB 8-week group (n = 5), TAA 12-week group (n = 5) and TAA + LAB 12-week group (n =5). The TAA 8-week and TAA + LAB 8-week groups were sacrificed at 8 weeks. The remaining rats were sacrificed at 12 weeks. At the time of sacrifice, blood and liver samples were harvested. Livers were fixed in 10% formalin or frozen in liquid nitrogen for histological analysis.

B. Histological and immunohistochemical examination

Four-micrometer-thick sections of formalin-fixed and paraffin-embedded livers from all experimental groups were processed for hematoxylin and eosin staining (H&E) and Masson's trichrome staining. Tissue sections were immunostained for α-SMA (Dako North America, Inc, Carpinteria, CA, USA) and diluted 1:1000. Stained liver sections were analyzed with the computerized imaging analysis system (MetaMorph version 4.6r5, Universal Imaging Corp, Downingtown, PA, USA).

C. Biochemical parameters

Rat whole blood was obtained at the time of sacrifice via vena cava puncture. After centrifugation of each blood sample, serum was obtained and examined for both aspartate aminotransferase (AST) and alanine aminotransferase (ALT).Rat whole blood was obtained at the time of sacrifice via vena cava puncture. After centrifuge of each blood sample, serums were obtained and examined for both aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

D. Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted from frozen rat liver tissues, using Takara RNA PCR (AMV) Ver 3.0 (Takara Bio Inc, Shiga, Japan) as described in the product protocol, and cDNA was generated from 1 μg of total RNA, using oligo dT-Adaptor primer and Avian Myeloblastosis Virus reverse transcriptase (Life Science, Boston, MA, USA). Real-time PCR was performed on Roche LightCycler® (Roche Diagnostics GmbH, Mannheim, Germany). PCR primers for transforming growth factor (TGF)-β were 5'-CCTGGAAAGGGCTCAACAC-3' sense and 5'-CAGTTCTTCTCTGTGGAGCTGA-3' antisense. PCR primers for α-SMA were 5'-CGATAGAACACGGCATCATCAC-3' sense and 5'-GCATAGCCCTCATAGATAGGCA-3' antisense. PCR primers for type I collagen α1 were 5'-CATGTTCAGCTTTGTGGACCT-3' sense and 5'-GCAGCTGACTTCAGGGATGT-3' antisense. The relative expression of target gene mRNA was normalized to the amount of rat porphobilinogen deaminase (PBGD) mRNA in an identical cDNA sample.[17] PCR primers for PBGD were 5'-CACCTGGAATTCAAGAGTATTCG-3' sense and 5'-CCAGGATAATGGCACTGAACT-3'.

3. *In vitro* analysis

A. Cell culture

The immortal human HSC cell line established by functional expression of the telomerase catalytic subunit (human telomerase reverse transcriptase or hTERT) was used in this study.[18,19]

Both primary cultured cells and hTERT-HSCs were cultured in a 5% CO_2 humidified incubator at 37℃. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% streptomycin-penicillin mixture. Before the start of all experiments, the cells were cultured with DMEM without containing phenol red and FBS for 24 hours (serum starvation).

B. Cell viability and proliferation assay

After hTERT-HSCs were plated in a 24-well microplate (Nunc™, Roskilde, Denmark) at a density of 3×10^4 cells/well in complete culture medium, cell cytotoxicity was estimated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. For cell proliferation assay, the cells were incubated with platelet-derived growth factor (PDGF) BB (R&D systems,

Minneapolis, MN, USA) with/without LAB for 48 hours before MTT assay. The number of viable cells was measured at a wavelength of 540 nm on an enzyme-linked immunosorbent reader, VERSAmax™ (Molecular Devices, Sunnyvale, CA, USA).

C. Measurement of reactive oxygen species

Cells at a density of 2×10^4 cells/well in 96-well plates were incubated with LAB for 24 hours and then loaded with a redox-sensitive dye, 50 μM H_2DCFDA (Molecular Probes, Eugene, OR, USA) at 37℃ for 30 minutes. The cells were rinsed twice with phosphate buffered saline (PBS) and stimulated with 100 μM hydrogen peroxide (H_2O_2). The fluorescence was detected at an excitation wavelength of 495 nm and an emission wavelength of 529 nm. ROS formation was measured using Luminescence Spectrometer LS50B (Perkin-Elmer Corp, Norwalk, CT, USA).

D. NF-κB responsive luciferase assay

Recombinant adenoviral vectors expressing a luciferase reporter gene driven by NF-κB transcriptional activation (Ad5NF-κBLuc) were used for the assessment of NF-κB transcriptional activation.[19] HSCs were infected with

Ad5NF-κBLuc for 12 hours in DMEM containing 0.5% FBS. After infection, the culture medium was changed to fresh medium with 0.5% FBS, and the culture was continued for an additional 8 hours. At 20 hour post infection, HSCs were stimulated with tumor necrosis factor-α (TNF-α) for 8 hours in serum-free conditions with or without pretreatment with LAB. A luciferase assay kit with luciferase cell culture lysis buffer (Promega, Madison, WI, USA) was used to measure NF-κB mediated transcriptional induction according to the manufacturer's protocol.

E. Western blots

The stimulant was added for 30 minutes to HSCs after 24 hours of preincubation with and without 40 μM LAB. Whole-cell extracts were prepared using Triton lysis buffer containing protease and phosphatase inhibitors. Forty micrograms of protein was electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. The gels were blotted onto a nitrocellulose membrane. Antibodies against phospho-p44/42 MAP kinase (Cell Signaling Technology, Inc, Beverly, MA, USA) and α-tubulin, all diluted 1:1000, were used.

For secreted type I collagen measurement, 0.76 g of sodium sulfite (Na_2SO_3) was added to 4 mL of supernatant from each well after 48 hours of incubation with LAB. The precipitate after centrifugation was resuspended with 0.5 M

acetic acid. Aliquots of 40 μL were electrophoresed and blotted the same as described above. Antibodies against collagen type I (Biodesign international, Saco, ME, USA), diluted 1:1000, were used.

4. Statistics

The data are shown as mean ± standard deviation. They were analyzed with the Wilcoxon rank sum test. A *P* value of < 0.05 was considered statistically significant.

III. RESULTS

1. *In vivo* analysis

The representative photographs of the liver are shown in Fig. 1. Diffuse granularity of the liver surface was noted in rats treated with TAA 8 for weeks (Fig. 1-C). This change was attenuated by LAB (Fig. 1-D). Diffuse micronodularity with shrinkage of the liver was noted in rats treated with TAA for 12 weeks (Fig. 1-E). This cirrhotic change was also attenuated by LAB (Fig. 1-F). LAB-treated rats without TAA did not demonstrate any gross change of the liver.

Liver sections were stained for Masson's trichrome and immunostained for α-SMA. Representative photomicrographs are shown in Fig. 2. Fibrous septa developed in rats treated with TAA for 8 weeks, incompletely surrounding regenerative parenchyma. Partial nodular formation was present without obvious cirrhosis (Fig. 2-A). This incomplete septal fibrosis was attenuated by LAB (Fig. 2-B). Diffuse nodules of varying size with fibrous septa were noted in rats treated with TAA for 12 weeks, and this overt cirrhotic change was also attenuated by LAB (Fig. 2-C & D). α-SMA positive cells were evident on the periphery of regenerating nodules in rats treated with TAA (Fig. 2-E & G). However, the expression of α-SMA was significantly decreased in rats treated with TAA + LAB (Fig. 2-F & H).

Stained-area analyses using computerized imaging analysis system are shown in Table 1. LAB decreased TAA-induced hepatic fibrosis in sections stained for Masson's trichrome. LAB also decreased TAA induced HSC activation as shown in liver sections immunostained for α-SMA.

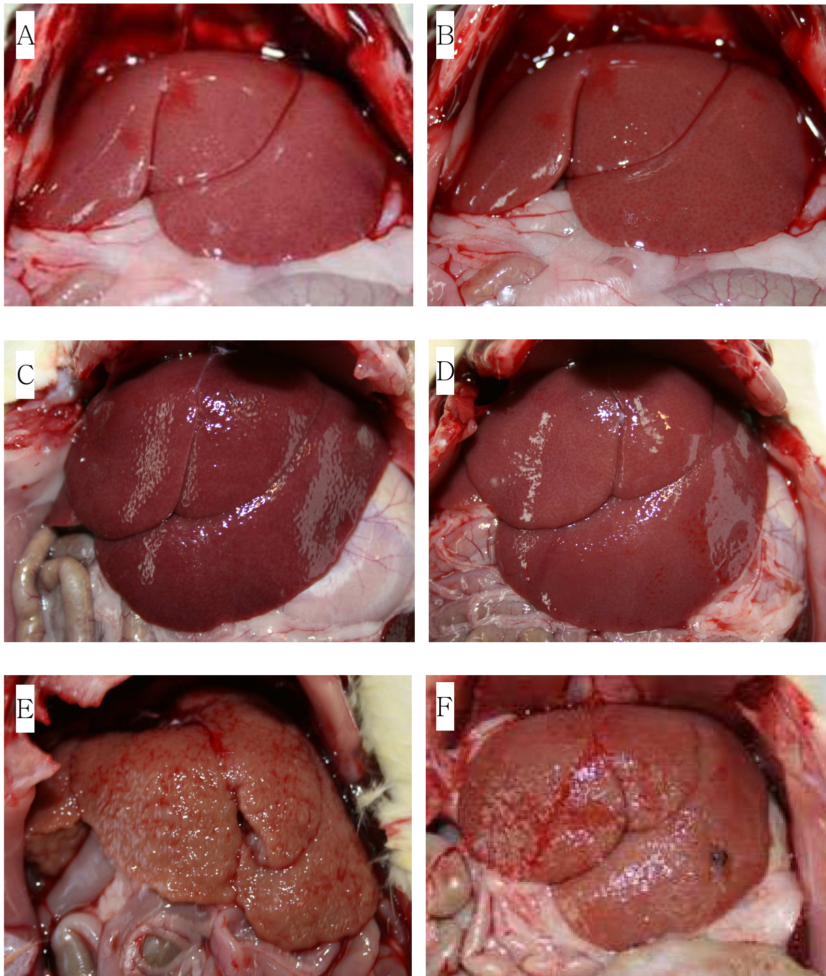

Fig. 1. Gross appearance of the liver. (A) The control group. (B) The control + LAB group. (C) The TAA group sacrificed at 8 weeks. (D) The TAA + LAB group sacrificed at 8 weeks. (E) The TAA group sacrificed at 12 weeks. (F) The TAA + LAB group sacrificed at 12 weeks.

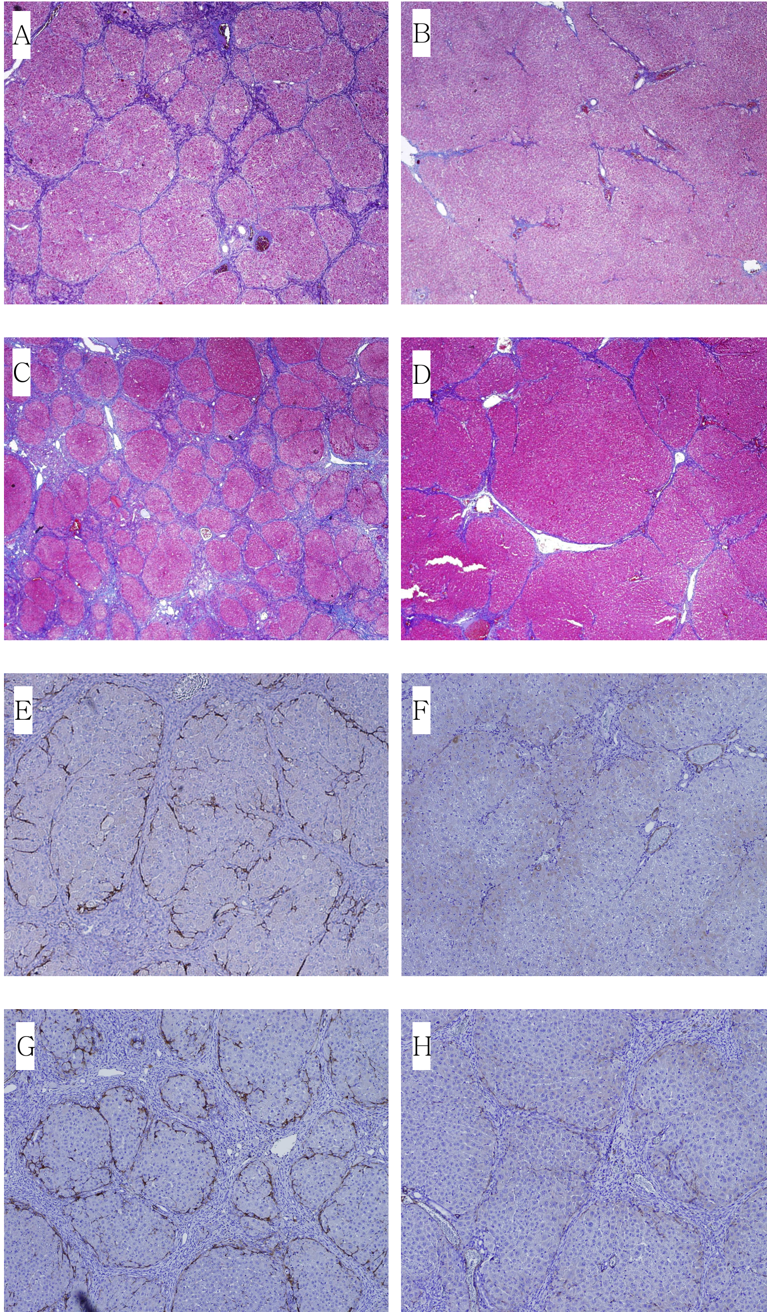

Fig. 2. Microscopic findings of liver sections. Representative photomicrographs

of liver sections were processed for Masson's trichrome stain (A, B, C and D; X40) and for immunohistochemical staining for α-SMA (E, F, G and H; X40). (A) and (E) Liver sections of rats treated with TAA for 8 weeks. (B) and (F) Liver sections of rats treated with TAA + LAB for 8 weeks. (C) and (G) Liver sections of rats treated with TAA for 12 weeks. (D) and (H) Liver sections of rats treated with TAA + LAB.

Table 1. Percent stained area of liver sections

Group	Masson's trichrome stain	α-SMA immunostain
Control	1.2 ± 0.4	0.2 ± 0.1
Control + LAB	1.1 ± 0.4	0.1 ± 0.1
TAA 8-week	8.3 ± 1.8	2.1 ± 0.8
TAA + LAB 8-week	4.5 ± 2.5*	0.6 ± 0.1*
TAA 12-week	15.4 ± 1.5	2.1 ± 0.5
TAA + LAB 12-week	10.6 ± 1.8†	1.7 ± 0.5

*$P < 0.05$ compared with TAA 8-week.

†$P < 0.05$ compared with TAA 12-week.

Biochemical parameters were analyzed. The levels of AST and ALT were significantly higher in the TAA 8-week group than those in the TAA + LAB 8-week group (Table 2). The AST level in the TAA 8-week group was 280.5 ± 119.9 IU/L, whereas it was 114.0 ± 12.2 IU/L in the TAA + LAB 8-week group. Likewise, the ALT level in the TAA 8-week group was 100.0 ± 17.9 IU/L, whereas it was 71.5 ± 9.3 IU/L in the TAA + LAB 8-week group. However, the levels of these parameters were not significantly different after 12-week treatment of TAA with and without LAB. The levels of AST and ALT were not significantly different between control and control + LAB group.

Table 2. Biochemical parameters

Group	AST (IU/L)	ALT (IU/L)
Control	112.5 ± 24.9	46.8 ± 4.9
Control + LAB	114.5 ± 16.5	50.5 ± 6.2
TAA 8-week	280.5 ± 119.9	100.0 ± 17.9
TAA + LAB 8-week	114.0 ± 12.2*	71.5 ± 9.3*
TAA 12-week	232.5 ± 46.8	79.0 ± 10.1
TAA + LAB 12-week	245.8 ± 76.4	92.5 ± 21.3

*$P < 0.05$ compared with TAA 8-week.

Table 3. Relative mRNA expressions of α-SMA, TGF-β and type I collagen α1

Group	α-SMA	TGF-β	type I collagen α1
Control	1.0	1.0	1.0
Control + LAB	7.7 ± 1.0	1.1 ± 0.2	0.8 ± 0.2
TAA 8-week	52.3 ± 14.3	1.9 ± 0.4	19.6 ± 5.6
TAA + LAB 8-week	19.4 ± 12.9*	1.0 ± 0.2*	6.6 ± 1.0*
TAA 12-week	107.6 ± 40.1	4.0 ± 0.8	26.9 ± 4.4
TAA + LAB 12-week	18.4 ± 4.8†	1.5 ± 0.4†	21.2 ± 5.7

*$P < 0.05$ compared with TAA 8-week.

†$P < 0.05$ compared with TAA 12-week.

The relative expression of mRNA normalized to the amount of rat PBGD mRNA. Treatment with LAB significantly reduced target gene expression (Table 3). The relative induction of α-SMA, TGF-β and type I collagen α1 gene was increased by treatment with TAA. Significant decreases in individual gene expressions were noted in the TAA + LAB 8-week group compared to the TAA 8-week group. LAB significantly reduced the relative induction of mRNA of α-SMA and TGF-β after 12-week treatment. However, the relative expression of mRNA of type I collagen α1 between the TAA 12-week and TAA + LAB 12-week groups were not significantly different.

2. *In vitro* analysis

Treatment with up to 40 μM LAB did not reveal any discernable cytotoxicity (Fig. 3).The estimated cell viability by MTT assay after 72 hours treatment with 40 μM LAB was 95.6% ± 8.3%.

Cell proliferation measured by MTT assay after 48 hours is shown in Fig. 4. Cell viability of HSCs treated with 20 ng/mL PDGF alone was 223.7% ± 13.8%, which was significantly higher than that of HSCs which were not treated (No Tx) (P < 0.001). Cell viability of HSCs treated with 20 ng/mL PDGF and 40 μM LAB was 162.5% ±14.4%, which was significantly lower than that of HSCs treated with 20 ng/mL PDGF alone (P < 0.001).

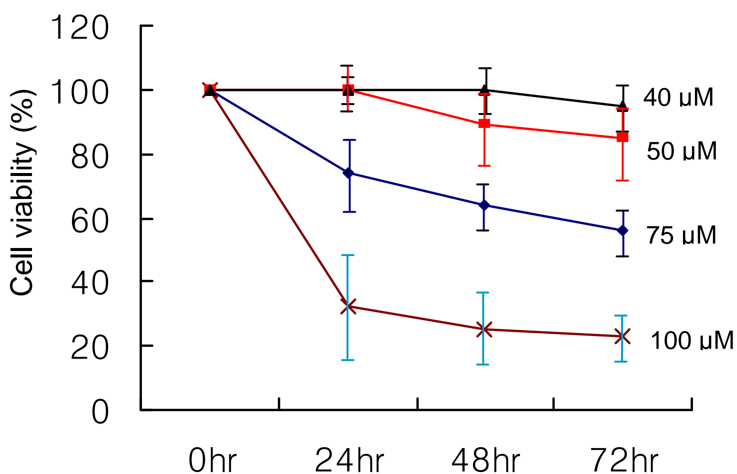

Fig. 3. Estimation of cell viability by MTT assay. Treatment with up to 40 μM LAB did not show any discernable toxicity.

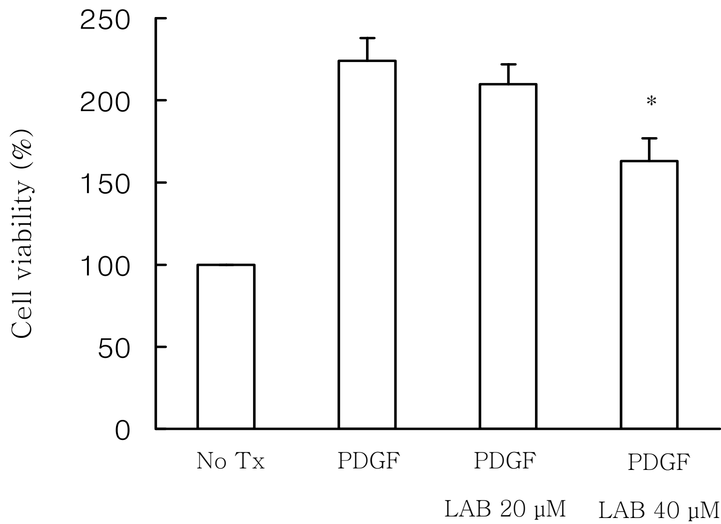

Fig. 4. PDGF-induced HSC proliferation was suppressed by LAB. HSCs were incubated for 48 hours. Cell viability of HSCs incubated with 20 ng/mL PDGF and 40 μM LAB was significantly lower than that of HSCs incubated with 20 ng/mL PDGF alone.

$^{*}P < 0.05$ compared with PDGF treatment.

Intracellular ROS was measured using 50 μM H_2DCFDA labeling. While 100 μM H_2O_2 markedly increased ROS formation, cells preincubated with 40 μM LAB showed markedly reduced ROS formation after 100 μM H_2O_2 stimulation (Fig. 5).

NF-κB transcriptional activation was evaluated by estimating the NF-κB-

mediated luciferase activity. While 10 ng/mL TNF-α induced NF-κB transcriptional activation (Fig. 6), pretreatment with LAB decreased NF-κB-mediated luciferase activity.

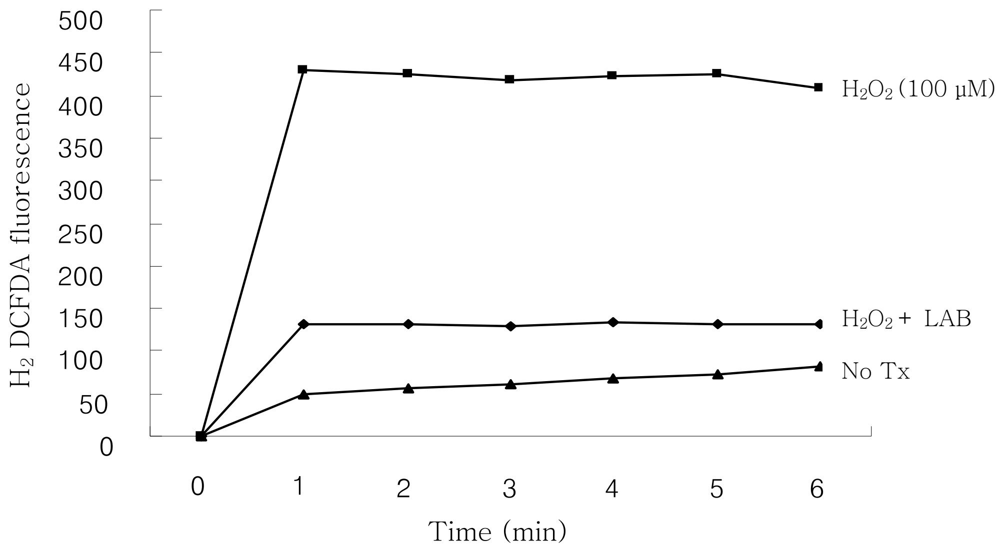

Fig. 5. LAB decreased ROS formation in HSCs. HSCs were preincubated with or without 40 μM LAB and loaded with 50 μM H_2DCFDA for 30 minutes. Cells were stimulated with 100 μM H_2O_2, and fluorescence was measured with a spectrometer.

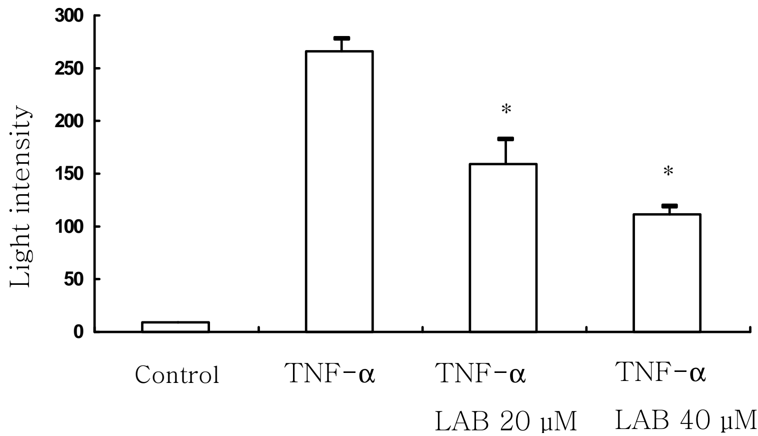

Fig. 6. LAB attenuated NF-κB transcriptional activation in HSCs. TNF-α (10 ng/mL) induced NF-κB transcriptional activation in HSCs. Whereas, HSCs pretreated with LAB showed a decrease in luciferase activity after TNF-α stimulation.

*$P < 0.05$ compared with TNF-α treatment.

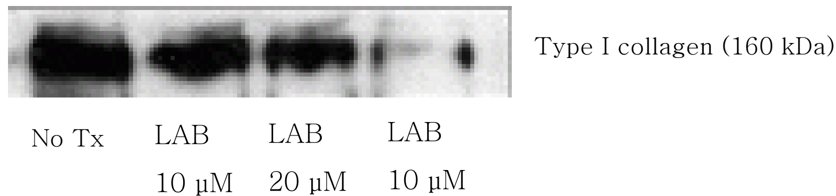

Fig. 7. LAB inhibited secretion of type I collagen. Type I collagen in the media was precipitated with Na_2SO_3 after incubation with LAB for 48 hours. The precipitate was centrifuged and resuspended in 0.5 M acetic acid. Then, aliquots of 40 μL underwent the immunoblot process.

Secretion of type I collagen was inhibited by 40 μM LAB (Fig. 7). Type I collagen production was markedly reduced in HSCs with incubated with 40 μM LAB for 48 hours.

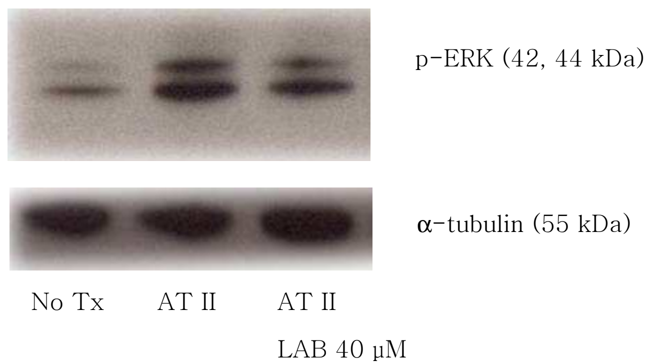

Fig. 8. LAB attenuated p-ERK expression in HSCs. Angiotensin II stimulated phosphorylation of ERK. HSCs preincubated with LAB decreased p-ERK expression compared to HSCs treated with AT II.

Phosphorylation of ERK was stimulated by 10^{-6} M angiotensin II (AT II). HSCs preincubated with 40 μM LAB showed a decrease in p-ERK expression compared to HSCs with AT II treatment.

IV. DISCUSSION

In the *in vivo* study, rats treated with TAA were sacrificed at 8 and 12 weeks.

Rats sacrificed at 8 weeks showed the early stage of cirrhosis, and rats sacrificed at 12 weeks showed an overt cirrhosis. LAB treatment attenuated liver fibrosis at 8 and 12 weeks in view of Masson's trichrome stain. However, the area of immunostain for α-SMA was not different between the TAA 12-week and TAA + LAB 12-week groups.

Results of AST and ALT demonstrated similar patterns. At 8 weeks, LAB attenuated inflammation in view of AST and ALT. However, these effects deteriorated at 12 weeks.

The relative mRNA levels of target genes were evaluated. At 8 weeks, the effect of LAB was prominent in that the relative induction of α-SMA, TGF-β, and type I collagen α1 were inhibited by LAB treatment. However, the effect of LAB was blunted in that the mRNA level of type I collagen α1 was not significantly different between the TAA 12-week and TAA + LAB 12-week groups.

Taken together, the effects of LAB were prominent and consistent in the early stage of cirrhosis, but these effects were offset by TAA in the late stage of cirrhosis.

TAA is a potent hepatotoxin. It is transformed into TAA sulfoxide or TAA sulfone by cytochrome P 450 and induces oxidative damage to the liver.[20] In this study, LAB demonstrated an antifibrotic effect in TAA-treated rats. These

results suggested that LAB attenuated the fibrogenic process in tissues probably by antioxidant activity. Our *in vivo* study suggested that HSC activation was suppressed by LAB. Consequently, antifibrogenic and inflammatory properties of LAB were identified.

Based on the *in vivo* results, the antifibrogenic mechanism of LAB was evaluated in the human HSC cell line. In the *in vitro* study, PDGF-induced HSC proliferation was suppressed by LAB. HSC activation is of crucial importance in hepatic fibrosis. The fundamental features of HSC activation seem to be similar irrespective of the initial cause of injury.[1] PDGF appears to be the most potent mitogen of HSCs during hepatic fibrogenesis.[20] In response to PDGF, the ERK pathway is activated in cultured human HSCs.[22] This pathway is necessary for PDGF-induced cell proliferation.[22] AT II is also a pro-oxidant and a fibrogenic cytokine.[23] AT II increases ROS formation and stimulates ERK in primary hepatocytes.[24] In this study, AT II stimulated phosphorylation of ERK, and LAB attenuated this process. These results indicated that LAB attenuated HSC proliferation by blocking the ERK pathway.

Oxidative stress regulates liver damage by altering the signal transduction pathway.[25] The MAPK and NF-κB pathways are implicated in hepatocyte damage from oxidative stress.[8] MAPKs regulate cellular functions including proliferation and differentiation.[8] NF-κB regulates the expression of

antiapoptotic genes.[17] H_2O_2-induced ROS formation in HSCs was decreased by LAB. Also, NF-κB transcriptional activation was suppressed by LAB treatment. These results can also validate the antioxidant property of LAB. Consequently, collagen production in HSCs was inhibited by LAB treatment. Western blot demonstrated that HSCs treated with LAB secreted much less type I collagen compared to HSCs with no treatment.

To date, numerous compounds with antifibrotic effects have been demonstrated in experimental models of fibrosis.[26] These pharmacological interventions included various stages of fibrogenesis including fibroprevention, fibrostasis and fibrolysis.[5] However, there has been no antifibrotic agent that has been widely used clinically. LAB exerted an antifibrotic effect in human HSCs and TAA-induced cirrhotic rats. LAB attenuated activation of HSCs and collagen production in *in vivo* and *in vitro* experiments. These antifibrogenic properties of LAB are most likely due to antioxidant activity.

V. CONCLUSION

In vitro analysis also focused on HSC activation and consequent collagen production. Furthermore, signal transduction pathways involved in oxidative

damage were investigated.

PDGF-induced proliferation of HSC was suppressed by LAB. Collagen production was also suppressed by LAB treatment. p-ERK expression was attenuated by LAB and NF-κB transcriptional activation was also attenuated by LAB. These two signal transduction pathways are implicated in hepatocyte injury from oxidative stress. H_2O_2-induced proliferation of HSCs was suppressed by LAB. Therefore, it might be concluded that LAB demonstrated anti-fibrogenic effects in HSCs by anti-oxidant activity.

In conclusion, LAB revealed anti-fibrogenic effect in human HSCs and TAA-induced rats. These anti-fibrogenic effects resulted from anti-oxidant property of LAB. Hepatic fibrosis refers to a wound-healing response to a variety of insults, ultimately leading to cirrhosis. The accumulation of ECM following liver damage is of paramount importance. Numerous compounds with antifibrotic effects have been tested in experimental models of fibrosis. However, these agents have not yet been effective in clinical trials.

LAB, one of the major active components from *Salvia miltiorrhizae*, was tested as antifibrogenic agent in this study. A TAA-treated rat model was used to evaluate the *in vivo* effect of LAB, and the *in vitro* effect of LAB was evaluated in the human HSC cell line. LAB attenuated the fibrogenic process and suppressed activation of stellate cells in liver sections. Masson's trichrome stain

and immunostain for α-SMA demonstrated the effects of LAB on liver tissues. The relative induction of mRNA α-SMA, TGF-β and type I collagen α1 were suppressed in liver tissues with LAB treatment. It is concluded that HSC activation and consequent collagen production may be suppressed in liver tissues with LAB treatment. Also, inflammatory responses were attenuated in rats with LAB treatment. Based on the fact that TAA causes oxidant damage to the liver and that LAB attenuated TAA-induced hepatic fibrosis, it seems that LAB has an antioxidant property.

In vitro analysis also focused on HSC activation and consequent collagen production. Furthermore, signal transduction pathways involved in oxidative damage were investigated. PDGF-induced HSC proliferation was suppressed by LAB. Collagen production was also suppressed by LAB treatment. Both p-ERK expression and NF-κB transcriptional activation were attenuated by LAB. These 2 signal transduction pathways are implicated in hepatocyte damage from oxidative stress. H_2O_2-induced HSC proliferation was suppressed by LAB. Therefore, it is concluded that LAB may exert antifibrogenic effects in human HSCs and TAA-induced rats by antioxidant activity.

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Abstract (in korean)

사람 간성상세포주 및 백서에서 Magnesium Lithospermate B의 항섬유화 효과

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간섬유화 현상은 간 조직에서 나타나는 상처치유 과정의 일종으로서 거듭되는 간조직의 손상과 재생과정을 통하여 간경변증이 발생되고 있다. 현재까지 수많은 항섬유화 치료가 시도되었으나 효과적인 항섬유화 약제로 사용되는 물질은 없다.

약초의 일종인 *Salvia miltiorrhiza*의 추출물은 페놀화합물로 구성되어 있으며 중국에서 혈관계통의 질환 치료에 널리 이용되고 있다. *Salvia miltiorrhiza*의 주요 성분중의 하나인 magnesium lithospermate B (LAB)는 항산화 효과로 신장보호 성질을 가지고 있다고 알려져 있다.

이에 저자는 사람 간성상세포주 및 백서에서 LAB의 항섬유화

효과를 알아보고자 하였다. 동물 간섬유화 모델은 thioacetamide (TAA) 랫드 모델을 사용하였다. 사람 간성상세포주는 정상 사람 간조직으로부터 간성상세포를 분리한 뒤에 레트로바이러스를 이용하여 불멸화시킨 human telomerase reverse transcriptase (hTERT) 간성상세포주를 사용하였다.

랫드는 각 군당 5마리씩 6개 군으로 분류하였다. 대조군 5마리, LAB투여군 5마리, TAA 투여군 10마리, TAA와 LAB 투여군 10마리로 하여 TAA 투여군 5마리와 TAA 및 LAB 동시 투여군 5마리는 8주에 희생하였으며 나머지는 12주에 희생하였다. 간조직을 Masson's trichrome 염색과 α-smooth muscle actin (α-SMA) 면역화학 염색을 시행 후 컴퓨터 이미지 분석을 통하여 염색된 면적을 구하였다. 간 손상 정도를 측정하기 위하여 랫드 희생 시 수집한 혈액에서 aspartate aminotransferase (AST)와 alanine transferase (ALT)를 측정하였다. 역전사효소 중합효소연쇄반응을 이용하여 α-SMA, transforming growth factor-β (TGF-β)와 type I collagen α1의 mRNA 수치를 상대적으로 측정하였다.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)를 이용하여 LAB의 세포독성 및 세포 증식 억제 효과를 측정하였다. 과산화수소 처리 후 발생하는 세포 내 반응성 산소기 억제 효과를 측정하였으며 tumor necrosis factor-α (TNF-α)를 처리 후 nuclear factor-κB (NF-κB)의 전사 활성도의 억제 정도를 측정하였다. 간성상세포에서 분비한 I형 교원질 및 안지오텐신 II에 처리 후 extracellular signal-regulated kinases 1/2 (ERK 1/2)의 표현이 LAB에 의해 억제되는지 측정하였다.

면역화학염색 결과 TAA 단독 투여 군에 비하여 TAA와 LAB를 동시에 투여한 군의 염색된 면적이 유의하게 낮았다. 8주 TAA군에

비하여 8주 TAA와 LAB 동시 투여군에서 AST, ALT 모두 의미 있게 낮은 수치를 보였으나 12주 TAA군과 12주 TAA와 LAB 동시 투여군에서는 의미 있는 차이가 없었다. α-SMA, TGF-β와 type I collagen α1의 상대적 mRNA 수치는 TAA군에 비해 TAA와 LAB 동시 투여군에서 유의하게 낮은 결과를 보였다.

MTT를 이용한 간성상세포 독성 실험 결과 LAB 40 μM까지는 세포 독성을 보이지 않았다. PDGF 및 LAB를 동시에 투여한 간성상세포는 PDGF 단독 투여한 세포에 비하여 증식이 억제되었다. 과산화수소로 자극하여 세포내 반응성 산소기를 측정한 결과 LAB로 전 처리한 간성상세포가 전 처리하지 않은 세포에 비하여 반응성 산소기가 의미있게 억제되는 소견을 보였다. TNF-α를 처리하였을 때 간성상세포에서 NF-κB의 전사 활성도가 증가하였으며 LAB를 전 처리한 간성상세포에서는 NF-κB의 전사 활성도가 의미 있게 감소함을 보였다. Western blot 결과 LAB를 처리한 간성상세포에서 분비한 I형 교원질의 양이 처리하지 않은 세포와 비교하여 적게 나타났다. 안지오텐신 II가 ERK 1/2의 인산화를 유도하였으며 LAB를 전처리한 간성상세포에서는 ERK 1/2의 표현이 감소되었다.

LAB는 항산화 작용으로 ERK 경로 및 NF-κB 신호전달체계 차단하여 사람 간성상세포 증식 및 교원질 생성을 억제하였으며 TAA로 유도한 랫드 간경변 모델에서 간섬유화 억제를 보였다. 결론적으로 사람 간성상세포주 및 랫드 간경변 모델에서 LAB의 항섬유화 효과를 확인하였다.

핵심되는 말 : 간섬유화, 간성상세포, magnesium lithospermate B, 항섬유화 약제