

Short Communication

The Inhibitory Effects of Aqueous Extract of *Magnolia officinalis* on Human Mesangial Cell Proliferation by Regulation of Platelet-Derived Growth Factor-BB and Transforming Growth Factor- β 1 Expression

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Abstract. Mesangial cell (MC) proliferation, mediated by platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)- β 1, and cyclin-dependent kinases (CDK), is the common feature of glomerulosclerosis. *Magnolia officinalis*, stem bark of *Machilus thunbergii* S., has multiple pharmacological effects. In this study, we investigated the influence of aqueous extract of *Magnolia officinalis* on MC proliferation, DNA synthesis, and expression of PDGF-BB, TGF- β 1, CDK1, CDK2, and CDK4 in fetal bovine serum (FBS)-activated human MC. *Magnolia officinalis* inhibited the MC proliferation, DNA synthesis, and the expression of PDGF-BB, CDK1, and CDK2 gene and CDK1, CDK2, and TGF- β 1 protein. These results suggest that the inhibitory effect of *Magnolia officinalis* on MC proliferation may be mediated by regulation of PDGF-BB and TGF- β 1 expressions and by modulation of CDK1 and CDK2 expression.

Keywords: *Magnolia officinalis*, mesangial cell, proliferation

Proliferation of mesangial cell (MC) and accumulation of extracellular matrix (ECM) proteins is the common pathohistological feature of glomerulosclerosis, which ultimately leads to renal failure. Numerous studies have implicated that platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)- β 1 are important regulators of MC proliferation and ECM expansion, and that cyclin-dependent kinases (CDK) are essential enzymes for the cell cycle progression in MC (1–3).

Magnolia officinalis, stem bark of *Machilus thunbergii* S., is one of the common used-herbs in East Asian countries and is prescribed in *Saiboku-to* which has prominent inhibitory effects on the proliferation of cultured murine MC (4). Recently, it was reported that *Perilla frutescens*, which is prescribed in *Saiboku-to*

together with *Magnolia officinalis*, has suppressive effects on mesangioproliferative glomerulonephritis in rats (5). Honokiol and magnolol, the main constituents of *Magnolia officinalis* have multiple pharmacological effects, such as anti-tumor, anti-oxidant, anti-thrombotic, anti-microbial, anti-allergic, anti-fungal, and anti-inflammatory effects (6). However, there is no study about the MC proliferation, especially with respect to growth factors and cell cycle, that could explain the suppressive mechanism, although the anti-proliferative effect of *Magnolia officinalis* in several cell lines has been reported (6). In this study, we evaluated the influence of an aqueous extract of *Magnolia officinalis* on cell proliferation; DNA synthesis; and expressions of PDGF-BB, TGF- β 1, CDK1, CDK2, and CDK4 in fetal bovine serum (FBS)-activated human MC.

The air-dried and crushed *Magnolia officinalis* (100 g) was added to distilled water, and extraction was

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performed by heating at 100°C; then the extract was concentrated with a rotary evaporator and lyophilized. The final aqueous extracts, weighing 6 g (a collection rate of 6%), was diluted with saline solution.

MCs were characterized as previously described (7). Normal renal cortex was obtained from a patient undergoing nephrectomy for a renal carcinoma. Glomeruli were separated from the cortex by sieving. Primary culture of MCs was established from collagenase-treated glomeruli. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented 15% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM L-glutamine, 2 mM sodium pyruvate, 1% (v/v) nonessential amino acids, 5 µg/ml transferrin, and 5 ng/ml selenium. Culture flasks were kept in a 5% CO₂ incubator. Experiments were performed on MCs of the third passage.

Cell viability was measured by a modified 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (7). MCs were seeded at a density of 1×10^4 cells/well in a 96-well plate and grown to confluence. The cells were added with the previous culture medium containing different concentrations of *Magnolia officinalis* and 250 µM captopril as the active-control for 48 h. Absorbance at 570 nm was read for each well using an ELISA reader.

DNA synthesis was measured as the incorporation of ³H-thymidine into trichloroacetic acid (TCA)-insoluble material (7). MCs were seeded into a 24-well dish at a density of 4×10^4 cells/well, and they were grown to confluence and then starved in serum free medium for 48 h. To test the suppressive effects of *Magnolia officinalis*, the cells were incubated with 25 or 50 µg/ml *Magnolia officinalis* and 250 µM captopril for 18 h, and then they were activated by 10% FBS for 28 h. During the last 4 h, cells were added with ³H-thymidine (1 uCi/ml; Amersham, Uppsala, Sweden). The incorporation of ³H-thymidine was counted in scintillation fluid using a β-counter. In the parallel, cell proliferation was measured by direct cell counting, as previously described (7).

To isolate RNA, MCs were plated in 10 cm² dishes at density of 1×10^6 cells/dish, grown to confluence, and rested in serum-free medium for 48 h. Then the cells were treated for 18 h with the 25 µg/ml *Magnolia officinalis* and 250 µM captopril. After activation with 10% FBS for 2 h, total RNA was isolated by RNA Zol B (TELTEST, Friendswood, TX, USA).

To evaluate the expression level of angiotensin converting enzyme (*ACE*) (8), *PDGF-BB* (7), *CDK1*, *CDK2*, and *CDK4* (9) mRNA, we performed a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The efficiency of the RT-PCR was

controlled by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification. PCR was carried out on 30-µl samples in a Gradient PCR device (Eppendorf, Hamburg, Germany). Each sample mixture contained standard PCR buffer, 2.5 mM dNTP, 2 U *Taq* polymerase, and 5 pM of each of the following primers: *ACE*, 5'-GCCTCCCCAACAAGACTGCCA-3' and 5'-CCACATGTCTCCCAGCAGATG-3'; *PDGF-BB*, 5'-GAAGGAGCCTGGGTTCCTG-3' and 5'-TTTCTCACTGGACAGGTCG-3'; *CDK1*, 5'-TCAAAGCTGGCTCTTGA-3' and 5'-CCTGGTTTCCATTTGGGA-3'; *CDK2*, 5'-GCTTTCTGCCATTCTCATCG-3' and 5'-GTCCCCAGAGTCCGAAAGAT-3'; *CDK4*, 5'-ACGGGTGTAAGTGCCATCTG-3' and 5'-TGGTGTCTGGTGCCTATGGGA-3'; *GAPDH*, 5'-TGGTATCGTGGAA GGA CT CAT GAC-3' and 5'-ATGCCAGTGAGCTTC CCGTTCAGC-3'. The PCR cycles consisted of steps at 94°C for 3 min, then 25 cycles (for *PDGF-BB*, *CDK4*, and *GAPDH*), 30 cycles (for *CDK1* and *CDK2*), and 42 cycles (for *ACE*) at 94°C for 45 s, 63°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. The expected PCR product size was 389 bp (for *ACE*), 226 bp (for *PDGF-BB*), 848 bp (for *CDK1*), 316 bp (for *CDK2*), 464 bp (for *CDK4*), and 450 bp (for *GAPDH*). The reaction products were subjected to computer-assisted densitometry after electrophoresis on a 2% agarose gel and staining with ethidium bromide.

For Western blot analysis of *CDK1* and *CDK2*, MCs treated as described above were collected. Protein was extracted from the collected MCs using a PRO-PREPTM protein extract kit (Intron Biotechnology, Sungnam, Korea), and its concentration was determined using a bicinchoninic acid kit (Sigma, St. Louis, MO, USA). Thirty micrograms of total post-lysate protein were mixed with 5 × sodium dodecyl sulfate (SDS) sample buffer [250 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, and 0.001% bromophenol blue] and heated at 100°C for 5 min. These samples were separated by means of electrophoresis to a 12% SDS-polyacrylamide gel and transferred from the gel onto an Immobilon membrane (Millipore, Bedford, MA, USA). Membranes were probed with rabbit anti-human *CDK1* antibody (dilution of 1:1000, Sigma) and *CDK2* (dilution of 1:1000, Sigma). The detection was performed using goat anti-rabbit antibody conjugated with horseradish peroxidase and the enhanced chemiluminescence system (Amersham).

To determine the TGF-β1 expression of MC, the concentrations of TGF-β1 contained in cell culture supernatant was measured by a human TGF-β1 ELISA assay kit (R&D Systems, Minneapolis, MN, USA). In brief, MCs were plated in a 24-well dish at density of 4×10^4 cells/dish, grown to confluence, and rested in

serum-free medium for 48 h. Then the cells were treated for 18 h with 25, 50 $\mu\text{g/ml}$ *Magnolia officinalis* and 250 μM captopril. After activation with 10% FBS for 2 h, the supernatants were harvested. Microplates, coated with recombinant human TGF- β 1 sRII, were prepared; and 200 μl of duplicate activated samples or standard dilutions of TGF- β 1 were applied, then followed by 200 μl of TGF- β 1 conjugate, 200 μl substrate solution, and 50 μl of stop solution. Absorbency was measured by an ELISA reader at 450 nm.

Statistical comparisons were performed by using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. All data are presented as the mean \pm S.D. All *P* values are two-tailed, and significance was taken at *P*<0.05.

In order to exclude the cytotoxic effect of *Magnolia officinalis* and to determine the experimental concentration, MTT activity was evaluated with medium containing the different concentrations of *Magnolia officinalis* for 48 h. There was no variation in the MTT activity in the absence and presence of *Magnolia officinalis* (0 $\mu\text{g/ml}$ *Magnolia officinalis*, 2.303 ± 0.012 ; 25 $\mu\text{g/ml}$ *Magnolia officinalis*, 2.245 ± 0.063 ; 50 $\mu\text{g/ml}$ *Magnolia officinalis*, 2.157 ± 0.103).

The anti-proliferative effect of *Magnolia officinalis* was evaluated on FBS-activated human MC. After being activated by 10% FBS, MC was proliferated to 171200 ± 8800 cells/well; and 25, 50 $\mu\text{g/ml}$ *Magnolia officinalis* and 250 μM captopril markedly inhibited the MC proliferation in a dose-dependent manner (96200 ± 8800 , 53300 ± 3100 , and 135600 ± 6200 cells/well, respectively) (Fig. 1A). Furthermore, *Magnolia officinalis* showed more inhibitory effect than captopril. Consecutively, we confirmed the inhibitory effect on DNA synthesis of *Magnolia officinalis*. The inhibition of DNA synthesis by *Magnolia officinalis* was corresponded with the results shown in cell proliferation (Fig. 1B).

To determine the possible action mechanism of *Magnolia officinalis* on MC proliferation, we evaluated the expression of *ACE*, *PDGF-BB*, *CDK1*, *CDK2*, and *CDK4* genes using semi-quantitative RT-PCR. The expressions of *CDK1* and *CDK2* protein were evaluated by Western blotting, and the synthesis of TGF- β 1 protein was determined by ELISA. Taking the mRNA level in the control as 1, *ACE* and *PDGF-BB* mRNA levels were significantly inhibited by 25 $\mu\text{g/ml}$ *Magnolia officinalis* and 250 μM captopril to the following levels: *ACE*, 0.535 ± 0.157 and 0.508 ± 0.143 ; *PDGF-BB*, 0.608 ± 0.042 and 0.882 ± 0.067 , respectively (Fig. 2: A and C). The expression of *CDK1* and *CDK2* gene also was reduced by *Magnolia officinalis* to 0.646 ± 0.246 and 0.888 ± 0.072 , respectively. The

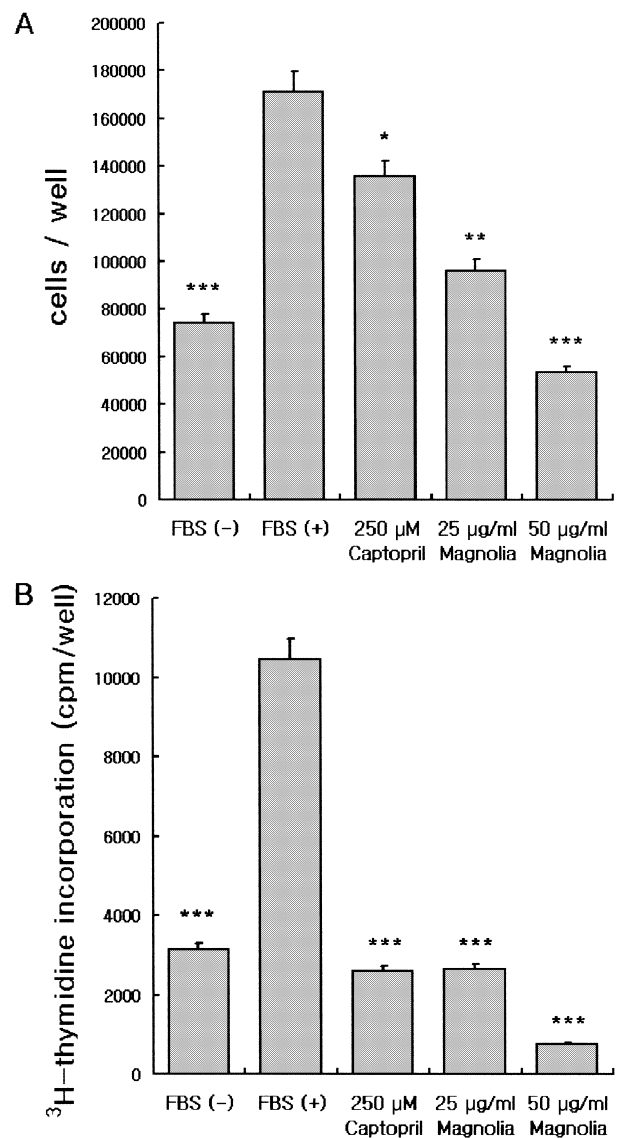


Fig. 1. Effect of *Magnolia officinalis* on FBS-activated mesangial cell proliferation and DNA synthesis. A: Mesangial cells were incubated with the 25 or 50 $\mu\text{g/ml}$ *Magnolia officinalis* and 250 μM captopril for 18 h, and then they were activated by 10% FBS for 48 h. The cell proliferation was measured by the direct cell counting. F-value in ANOVA was 488. B: In parallel, the amount of ^3H -thymidine incorporation into TCA was measured using a β -counter. F-value in ANOVA was 852. FBS (+) indicates 10% FBS-stimulated mesangial cells. Results are reported as the mean \pm S.D. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with FBS (+), each done in 4 separate experiments.

CDK4 mRNA level was not significantly changed by *Magnolia officinalis* (Fig. 2C). Consecutively, its inhibitory effect on *CDK1* and *CDK2* expression was confirmed using Western blot analysis. Taking the protein level in the control as 1, *CDK1* and *CDK2* protein levels were significantly inhibited by 25 $\mu\text{g/ml}$ *Magnolia officinalis* to the following levels: *CDK1*,

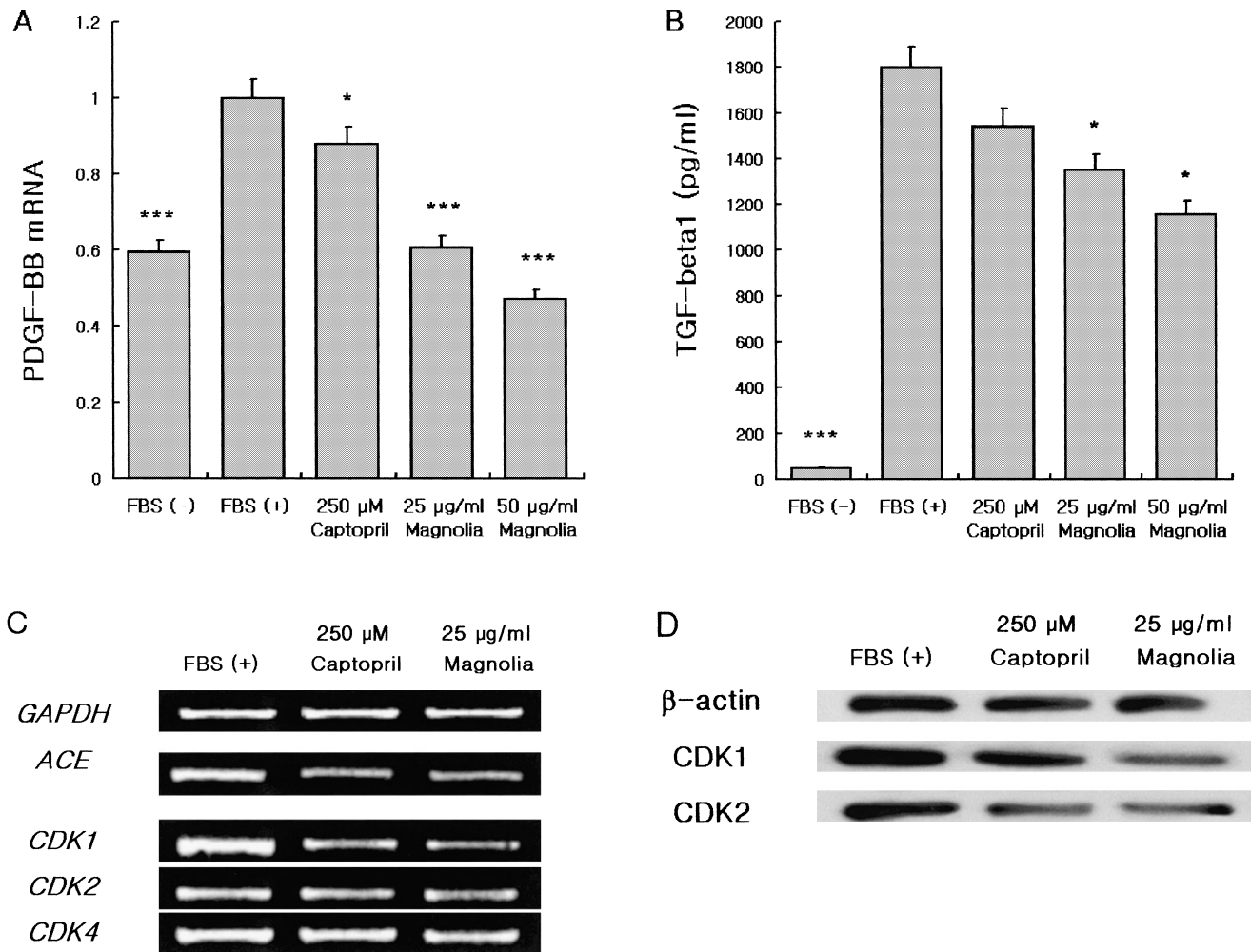


Fig. 2. Inhibitory effect of *Magnolia officinalis* on FBS-activated ACE, PDGF-BB, CDK1, CDK2, CDK4, and TGF- β 1. Mesangial cells were incubated with the 25 or 50 μ g/ml *Magnolia officinalis* and 250 μ M captopril for 18 h and then they were activated with 10% FBS for 2 h. Cells were collected, and total RNA was isolated for RT-PCR and protein, for Western blotting. Supernatants were harvested for TGF- β 1 ELISA assay. A: PDGF-BB mRNA level. Results of PDGF-BB were calculated by comparing them with the optical density of 10% FBS being taken as 1.00. F-value in ANOVA was 11. B: TGF- β 1 protein level. Results are reported as the mean \pm S.D. F-value in ANOVA was 191. C: GAPDH, ACE, CDK1, CDK2, and CDK4 mRNA expression. D: β -actin, CDK1, and CDK2 protein expression. FBS (+) indicates 10% FBS-stimulated mesangial cells. * P <0.05, *** P <0.001, compared with 10% FBS (+), each done in 3 separate experiments.

0.657 ± 0.078 ; CDK2, 0.746 ± 0.110 , respectively) (Fig. 2D) which corresponded with the results shown in RT-PCR. *Magnolia officinalis* significantly inhibited the expression of TGF- β 1 to 1353.479 ± 94.453 pg/ml at 25 μ g/ml and 1158.428 ± 123.529 pg/ml at a concentration of 50 μ g/ml, as compared with the control value of 1800.336 ± 113.187 pg/ml (Fig. 2B).

PDGF, in particular the PDGF-BB, has a strong mitogenic effect on MC proliferation (1), and TGF- β 1 regulates the production of proteoglycans, fibronectin (FN), and collagens in glomerular MC (2). The increasing productions of TGF- β , PDGF, and other factors including FN, type IV collagen, laminin, and so on have

been observed in FBS-stimulated MC proliferation and human IgA nephropathy (10, 11). Furthermore, administration of drugs to antagonize PDGF-BB and TGF- β 1 decreased MC proliferation and glomerulosclerosis in the experimental glomerulonephritic rats (12, 13).

Angiotensin II, which contribute to kidney diseases and their progression to end-stage renal disease, may up-regulate the expression of PDGF and TGF- β . An ACE inhibitor like captopril markedly decreases growth factor activation, consecutively reduces glomerulosclerosis and interstitial fibrosis (14), and slows the progression of renal function deterioration (15). In the present study, *Magnolia officinalis* showed an inhibitory effect on

the expression of ACE and growth factor, which may support that *Magnolia officinalis* traditionally has been used in progressive renal disease, and could prevent glomerulosclerosis by decreasing the growth factor activation like an ACE inhibitor does.

It has been reported that MC proliferation is associated with an increase in the expression of CDKs such as CDK2 (3). In present study, CDK1 and CDK2 expression was decreased by *Magnolia officinalis* during MC proliferation, although there was no significantly change in CDK4.

From these results, we assumed that the inhibitory effect of *Magnolia officinalis* on MC proliferation might be mediated by suppression of PDGF-BB and TGF- β 1 expressions and by regulation of CDK1 and CDK2 expression.

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