Magnesium lithospermate B suppressed the proliferation of mesangial cells

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Abstract

The effects of magnesium lithospermate B, a component of Salviae Miltiorrhizae Radix, on the proliferation of mesangial cells were determined in terms of the ^{3}H-thymidine uptake. When magnesium lithospermate B was added to the medium of mesangial cell culture, it markedly suppressed the proliferation of mesangial cells in a dose-dependent manner, indicating a direct corroboration of the renal effects.

Key words mesangial cell, proliferation, magnesium lithospermate B, mouse.

Abbreviations ACE, angiotensin converting enzyme ; ANF, atrial natriuretic factor ; D-val MEM, minimum essential medium with D-valine substituted for L-valine ; FCS, fetal calf serum ; GFR, glomerular filtration rate ; PBS, phosphate-buffered saline ; PGE_2, prostaglandin E_2 ; RBF, renal blood flow ; RPF, renal plasma flow ; TGF-β, transforming growth factor beta.

Introduction

Chronic glomerulonephritis, the most common renal disease, is histologically characterized by the proliferation of mesangial cells. Various studies have been carried out to elucidate the mechanisms of such proliferation. It has been suggested that platelets or factors derived from the monocytic system or phagocytes are deeply involved in the progression and aggravation of this disease, and many factors promoting the proliferation of mesangial cells have been found among these cells.[1,2] On the other hand, there is no sufficient information on factors suppressing the proliferation of mesangial cells ; only a few factors such as atrial natriuretic factor (ANF), cAMP, heparin (-like substance), prostaglandin E_2 (PGE_2) and transforming growth factor beta (TGF-β) are known.[3] Recently, Powell *et al.*[4] have reported that angiotensin converting enzyme (ACE) inhibitors, which are commonly used for the treatment of hypertension, suppress the proliferation of smooth muscle cells present in injured vascular walls, awakening expectation for their prophylactic effects on arteriosclerosis, cerebrovascular disorder and ischemic cardiac disease. Takama *et al.*[5] have also pointed out the possibility that ACE inhibitors not only improve systemic hemodynamics in glomerulonephritis through their hypotensive effect,but also act directly on glomeruli to suppress the proliferation of mesangial cells, preventing the progression of glomerulonephritis. We have previously demonstrated the magnesium lithospermate B, a component of Salviae Miltiorrhizae Radix (a traditional Chinese medicinal herb known as "Dan Shen"), exerts an action similar to that of captopril, and that this action is enhanced by the combined use of captopril.[6] Our findings suggest that magnesium lithospermate B also has some effect on mesangial cells. In this regard, the effects of

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magnesium lithospermate B on the proliferation of mesangial cells were examined in the present study.

Materials and Methods

Animals: BALB/c female mice were obtained from Nippon Clea (Osaka, Japan).

Medium and reagents: Minimum essential medium with D-valine substituted for L-valine (D-val MEM) was obtained from Sigma Chemical Co. (St. Louis, Mo). D-val MEM supplemented with 20 % fetal calf serum (FCS; Gibco, Gland Island, N.Y.), 100 U/ml penicillin and 100 μg/ml streptomycin was used for the culture of mesangial cells.

Isolation of mouse glomeruli: In comparison to the conventional method utilizing serial sieving, Iwano *et al.*[7] established a new and more efficient method for the isolation of glomeruli. A BALB/c mouse at 4 weeks of age was killed with CO_2 gas in a bag. Immediately after respiratory arrest, the mouse was laid on its back and the thorax opened. A syringe filled with phosphate-buffered saline (PBS) was inserted into the left ventricle. After the right ventricle was incised for drainage, the PBS was perfused slowly but constantly into the heart. After most of the blood had been flushed out, the renal cortices were removed. The renal cortices were minced into small pieces (2 mm^3) and treated with 4 mg/ml of collagenase type IV (Worthington Biochemical Co., Freehold, N. J.) at 37°C for 30 min. After agitation with a vortex mixer for 10 min, the cortices were centrifuged at 3,500 rpm at 4°C for 20 min over a discontinuous 30 / 50 / 60 % gradient of Percoll (Pharmacia Inc., Piscataway, N. J.) and the 30/50 % interface obtained. These procedures (treatment with collagenase, agitation and centrifugation) were performed three times and as a result the tubules were almost completely excluded. After washing with Hanks'solution (Foundation of Research Institute for Microbial Disease, Osaka, Japan), highly purified glomeruli (more than 95 %) were obtained.

Mesangial cell culture: Primary cultures of mesangial cells were obtained from outgrowths of isolated mouse renal glomeruli. Washed glomerular remnants were plated onto plastic Petri dishes (Nunc, Roskilde, Denmark) at a density of 200 remnants/cm^2 in 20 % FCS containing medium and incubated at 37 °C in a humidified 5 % CO_2 atmosphere. Under these conditions, a homogenous outgrowth of elongated or stellate cells appeared within five days and reached confluence within ten days. Passaged cells were obtained by incubating washed confluent monolayers with a solution of trypsin (0.5 mg/ml, Sigma) and EDTA (0.2 mg/ml) for 5 min at 37 °C. Cells after three passages are considered as vascular smooth muscle-like mesangial cells using morphological and immunohistochemical criteria.[8]

Proliferation assay: Mouse mesangial cells, after four passages, were seeded in a 96-well microtiter plate (Costar, Cambridge, Mass) at a density of 10^4 cells/well in 0.2 ml D-val MEM containing 20 % FCS. After 24 h, the medium was changed to D-val MEM without FCS. Following another 48-hour incubation, the cells were cultured with or without magnesium lithospermate B (from 8.45 to 270μM) in the medium containing 8 % FCS for 48 h. The cultures were pulsed with 1 μCi of ^{3}H-thymidine (15.7 Ci/mM, New England Nuclear, Boston, Mass.) over the last 12 h of the 48-hour culture. Cells were then harvested onto a glass filter paper by an automated cell harvester (Lab Mash Science Co., Tokyo, Japan). The radioactivity was measured with Beckman Scintillation Counter (Beckman Instruments Incs., Fullerton, Calif.). All assays were performed in triplicate.

Purification of magnesium lithospermate B from Salviae Miltiorrhizae Radix: As reported previously,[9,10] commercially available Salviae Miltiorrhizae Radix (*Salvia miltiorrhiza* BUNGE) (1.0 kg) produced in China was extracted twice with water (1.5 liters) at 80 °C. After removal of the insolubles by filtration, the filtrate was concentrated under reduced pressure (40°C) and subjected to MCI-gel CHP-20P (7.5 cm *i.d.* × 35 cm) column chromatography. After washing the column with water, elution with 50 % aqueous methanol yielded polyphenols (62 g), which were chromatographed over Sephadex LH-20 (5.0 cm

i.d. × 42 cm) with water containing increasing amounts of ethanol to afford three fractions ; fractions I (4.8 g), II (0.35 g) and III (5.9 g), and compound I (7.56 g). Fractions I and III were rechromatographed separately over a Sephadex LH-20 column using water as an eluent to yield compound 2 (1.98 g) and a further fraction of compound 1 (4.3 g), respectively. Compound 1 was identified as magnesium lithospermate B (Fig. 1) on the basis of ^{13}C nuclear magnetic resonance spectra, infrared spectra, negative fast - atom bombardment mass spectroscopy, proton nuclear magnetic resonance spectra, energy-dispersive X-ray analysis and other data.

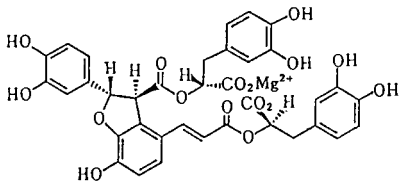

Fig. 1 Structural formula of magnesium lithospermate B.

Results

Figure 2 shows the proliferation activity of mesangial cells as determined in terms of the ^3H-thymidine uptake, using 10^4 mesangial cells per well. The ^3H-thymidine uptake by mesangial cells was 6510cpm in the absence of magnesium lithospermate B, whereas it was decreased by 20 % to 5240 in the presence of magnesium lithospermate B at a concentration of 8.45 μM. When the concentration of magnesium lithospermate B was increased to 33.8 μM, the ^3H-thymidine uptake further decreased by 59 % in comparison with the value obtained without magnesium lithospermate B. In the presence of magnesium lithospermate B 270 μM, the uptake was only 43 cpm, corresponding to 0.7 % of the uptake determined in the absence of magnesium lithospermate B. A trypan blue dye exclusion test revealed that the viability of cells after reaction was more than 90 %.

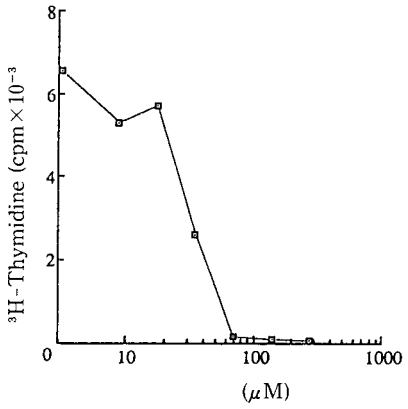

Fig. 2 Effect of magnesium lithospermate B on the proliferation of mesangial cells.

Discussion

It is said that most of urinary PGE_2 is derived from the kidney. The production of PGE_2 is known to take place in mesangial cells, glomerular epithelial cells, medullary interstitial cells, collecting tubules, *etc.*, in the kidney.[11] In particular, PGE_2 relaxes contracted mesangial cells and dilates glomerular capillaries to increase renal blood flow (RBF) and glomerular filtration rate (GFR), thereby improving renal function.[12] Magnesium lithospermate B, the substance tested in the present study, has previously been reported to induce a significant increase in urinary PGE_2 as well as GFR, renal plasma flow (RPF) and RBF after continuous intraperitoneal or oral administration.[13, 14] Magnesium lithospermate B has also been suggested to act on mesangial cells, since it has a hypotensive effect similar to that of ACE inhibitors in cases of renal hypertension or spontaneously hypertensive rats.[14, 15] In the present study, when magnesium lithospermate B was added to the medium of mesangial cell culture, it markedly suppressed the proliferation of mesangial cells in a dose-dependent manner. This suggests that magnesium lithospermate B is involved in the regulation of glomerular hemodynamics and the GFR through contraction and relaxation, i.e., probable physiological functions of mesangial cells. Thus, the present study provided direct

corroboration of the renal effects of magnesium lithospermate B suggested by the results of previous *in vivo* experiments. This is the first time that a component of an Oriental medicine had a renal effect of this type. Considering the fact that some Oriental medicines or medical prescriptions reportedly relieve renal disease, many other components similar to magnesium lithospermate B are expected to be contained in Oriental medicines, representing an interesting subject for future research.

和文抄録

糸球体腎炎の進行，増悪にメサンギウム細胞の増殖が深く関与していることが示されている。本研究ではメサンギウム細胞の増殖活性を ^{3}H-thymidine のとり込みを指標に検討した結果，magnesium lithospermate B 添加量の増加とともにメサンギウム細胞の増殖を著しく抑制する結果が得られた。このような作用を有する成分が和漢薬中から見い出されたのは初めてであり，*in vivo* 実験における magnesium lithospermate B の腎機能改善作用とも併せ，新しいタイプの和漢薬と考える。

References

1) Dubois, C.H., Foidart, J.B., Hautier, M.B., Dechenne, C. A., Lemaire, M.J. and Mahieu, P.R.: Proliferative glomerulonephritis in rats: evidence that mononuclear phagocytes infiltrating the glomeruli stimulate the proliferation of endothelial and mesangial cells. *Eur. J. Clin. Invest.* **11**, 91-104, 1981.

2) Cameron, J.S.: Platelets in glomerular disease. *Ann. Rev. Med.* **35**, 175-180, 1984.

3) Yamamoto, T.: Mesangial cell and cytokines. *Kidney and Dialysis* **28**, 643-650, 1990.

4) Powell, J.S., Clozel, J.P., Muller, R.K.M., Kuhn, H., Hefti, F., Hosang, M. and Baumgartner, H.R.: Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* **245**, 186-188, 1989.

5) Takama, T., Ochi, S., Yokoyama, K., Orita, Y. and Kamata, T.: Mesangial cell proliferation and angiotensin II. *Kidney and Dialysis* **28**, 651-655, 1990.

6) Yokozawa, T., Chung, H.Y., Lee, T.W., Oura, H., Nonaka, G. and Nishioka, I.: Potentiating effect of converting enzyme inhibitor captopril to the renal responses of magnesium lithospermate B in rats with adenine-induced renal failure. *Chem. Pharm. Bull.* **39**, 732-736, 1991.

7) Iwano, M., Dohi, K., Hirata, E., Horii, Y., Shiiki, H. and Ishikawa, H.: Induction of interleukin 6 synthesis in mouse glomeruli and cultured mesangial cells. *Nephron*, in press.

8) Striker, G.E. and Striker, L.J.: Biology of disease: Glomerular cell culture. *Lab. Invest.* **53**, 122-131, 1985.

9) Tanaka, T., Morimoto, S., Nonaka, G., Nishioka, I., Yokozawa, T., Chung, H.Y. and Oura, H.: Magnesium and ammonium-potassium lithospermates, the active principles having a uremia-preventive effect from *Salvia miltiorrhiza*. *Chem. Pharm. Bull.* **37**, 340-344, 1989.

10) Yokozawa, T., Chung, H.Y., Oura, H., Nonaka, G. and Nishioka, I.: Isolation of a renal function-facilitating constituent from the Oriental drug, Salviae Miltiorrhizae Radix. *Jpn. J. Nephrol.* **31**, 1091-1098, 1989.

11) Frölich, J.C., Wilson, T.W., Sweetman, B.J., Smigel, M., Nies, A.S., Carr, K., Watson, J.T. and Oates, J.A.: Urinary prostaglandins: Identification and origin. *J. Clin. Invest.* **55**, 763-770, 1975.

12) Serneri, G.G.N., Masotti, G. and Castellani, S.: Pathophysiology of renal prostaglandins. In "Contributions to Nephrology Vol. 49" (Ed. by G.M. Berlyne and S. Giovannetti), Karger, Basel, pp. 156-172, 1985.

13) Yokozawa, T., Lee, T.W., Chung, H.Y., Oura, H., Nonaka, G. and Nishioka, I.: Effect of magnesium lithospermate B on urinary prostaglandins in rats with renal failure. *J. Nat. Prod.* **53**, 662-665, 1990.

14) Yokozawa, T., Oura, H., Lee, T.W., Nonaka, G. and Nishioka, I.: Augmentation of renal response by magnesium lithospermate B. *Nephron* **57**, 78-83, 1991.

15) Zhang, X.Y., Yokozawa, T., Hattori, M., Oura, H., Nonaka, G. and Nishioka, I.: Hypotensive effect of magnesium lithospermate B. The 8th General Meeting of Medical and Pharmaceutical Society for WAKAN-YAKU, p. 136, 1991.