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Inhibition of collagen hydroxylation by lithospermic acid magnesium salt, a novel compound isolated from *Salviae miltiorrhizae* Radix

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Abstract

We have screened several chinese medicinal herbs for the presence of antifibrotic agents. An aqueous extract of *Salviae miltorrhizae* Radix was found to inhibit collagen secretion by human skin fibroblasts without affecting DNA or noncollagen protein synthesis. We have subsequently purified the material exhibiting the inhibitory activity and identified it as magnesium lithospermate. From its chemical structure this compound was predicted to be an inhibitor of the post-translational modifying enzymes prolyl and lysyl hydroxylases in collagen biosynthesis. Accordingly, it decreased the extent of prolyl and lysyl hydroxylations in collagen by approx. 50%. Added to cell extracts it inhibited both prolyl and lysyl hydroxylase activities, but only lysyl hydroxylase activity when added to intact cells. Oral administration of this compound to mice led to a significant reduction of prolyl hydroxylation in newly-synthesized skin collagen. This naturally-occurring compound thus offers a potential means for treating fibrotic diseases, such as systemic scleroderma and keloid.

Key words: Magnesium lithospermate; Collagen hydroxylation; Medicinal herb; Prolyl hydroxylase; Lysyl hydroxylase

1. Introduction

Synthesis of collagen, a major constituent of connective tissue, is controlled by numerous factors at the transcriptional or post-transcriptional level [1,2]. Overproduction of collagen is believed to be responsible for many of the clinical manifestations of fibrotic diseases such as systemic scleroderma [3], keloid [4], pulmonary fibrosis [5] and liver cirrhosis [6]. Although therapeutic interventions have been attempted, there is currently no suitable treatment for reducing excessive collagen deposition in these diseases.

Medicinal herbs have been used in China for over 1000 years for treating many diseases including fibrotic disorders in patients with systemic scleroderma. We have screened several Chinese medicinal herbs for the presence of antifibrotic agents and found one (*Salviae miltiorrhizae* Radix) containing a material identified as Mg lithospermate that specifically inhibited collagen secretion by human skin fibroblasts.

2. Materials and methods

Medicinal herbs, purchased from Osaka Funmatsu Yakuken Co. (Osaka, Japan), were finely powdered and extracted with methanol. The extracts were partitioned in water and ethyl acetate. The water- soluble fractions were used for screening. The medicinal herbs serving as the starting material were *S. miltiorrhiza, Rehmannia glutinosa, Zizyphus jujuba*, and *Persical semen*.

2.1. Fibroblast culture and metabolic labeling

Fibroblasts were explanted from apparent normal human skin by an established procedure. Cells were

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grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Confluent cultures treated for 48 h with aqueous extracts from medicinal herbs in DMEM supplemented with 0.5% FBS were labeled the last 18 h with [2,3-^{3}H]proline (50 μCi/ml; 53 Ci/mmol, Amersham) in DMEM supplemented with 0.5% FBS and ascorbic acid (30 μg/ml). For determination of the degree of prolyl and lysyl hydroxylations, cells were labeled the last 24 h with [2,3-^{3}H]proline (50 μCi/ml; 53 Ci/mmol, Amersham) and [U-^{14}C]lysine (2 μCi/ml; 400 μCi/mmol, Amersham) in lysine-free DMEM (Gibco). For determination of DNA synthesis, cells were labeled for 1 h with [*methyl*-^{3}H]thymidine (5 μCi/ml; 86 Ci/mmol, Amersham) in DMEM supplemented with 0.5% FBS. The amount of radioactivity incorporated in TCA-precipitable material was determined as described [7].

2.2. Determination of collagen synthesis

The amount of proline radioactivity incorporated into collagen was measured after digestion of the medium and cell layer separately with purified bacterial collagenase (Advanced Biofacture) as described [8].

2.3. Measurement of collagen hydroxylation

The proteins in the medium and cell layer were precipitated with 30% ammonium sulfate and separated by electrophoresis on a 4–15% gradient SDS-polyacrylamide gel. The procollagen bands were cut and hydrolyzed with 6 M HCl for 18 h at 110°C. The radioactive hydroxyproline, proline, lysine, and hydroxylysine were separated by HPLC and quantitated with a Beckman LS 1800 liquid scintillation spectrometer [9].

2.4. Purification of magnesium lithospermate from S. miltiorrhizae Radix

The water soluble fraction was chromatographed over a MCI-gel CHP-20P column (75–150 μm, Mitsubishi Chemical Industries) and eluted with 50% methanol. This fraction was further purified with Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals) chromatography as described [10]. Each fraction was assayed for collagen-suppressing activity in vitro.

2.5. Assay of prolyl and lysyl hydroxylase activities

Cell extracts were preincubated with 5 or 50 μg/ml of the compounds for 45 min at 25°C, then assayed [7] with an unhydroxylated procollagen substrate containing [4–^{3}H]proline for prolyl hydroxylase or [4,5–^{3}H]lysine for lysyl hydroxylase.

Fibroblast cultures were treated with various concentrations of the compounds for 48 or 72 h in DMEM supplemented with 0.5% FBS. The cell layer was trypsinized, extracted, and assayed for prolyl and lysyl hydroxylase activities [7].

2.6. Treatment of animals and extraction of newly synthesized collagen

Male hairless mice (10 wk of age) weighing approx. 50 g were fed on a commercial diet (type CE-2, CLEA Japan). After the adaptation period, a group of three mice was orally administered Mg lithospermate (1 mg/kg per day) for 30 days. Another group of three mice fed on a commercial diet alone was used as control. On the last day of treatment, [2,3–^{3}H]proline (200 μCi/50 g body wt.; 53 Ci/mmol, Amersham) was injected i.p. and allowed to label body proteins for 6 h. The skin from the dorsal side of the animal was then removed and homogenized with ice-cold 0.5 M acetic acid. The extraction was repeated four times. Acid soluble collagen was isolated with repeated salt precipitation at both neutral and acidic pH. The radioactivity in acid soluble collagen was counted. Acid soluble collagen was hydrolyzed with 6 M HCl for 18 h at 110°C and radioactive hydroxyproline and proline were separated and quantitated as described above.

3. Results

3.1. Inhibition of collagen production by S. miltiorrhizae Radix

One out of 4 medicinal herbs was found to inhibit collagen production by human skin fibroblasts (data not shown). We used an aqueous extract of this herb (*S. miltiorrhizae* Radix) for further studies and noted that it specifically decreased in a dose-dependent manner the appearance of newly synthesized collagen in culture medium with a maximal inhibition of 84% at 200 μg/ml (Fig. 1a), a concentration at which the amount of collagen in the cell layer increased 4-fold (Fig. 1b). The extract had no cytotoxic effect up to the concentration of 200 μg/ml, as indicated from its inability to significantly alter thymidine incorporation (Fig. 1c). The simplest explanation for these observations would be that the extract inhibited procollagen secretion through its effect on prolyl hydroxylation. To test this, we measured the degree of collagen hydroxylation in drug-treated cells. Both prolyl and lysyl hydroxylations in procollagens (mainly type I) isolated from SDS-polyacrylamide gels were reduced 40–56% (Table 1).

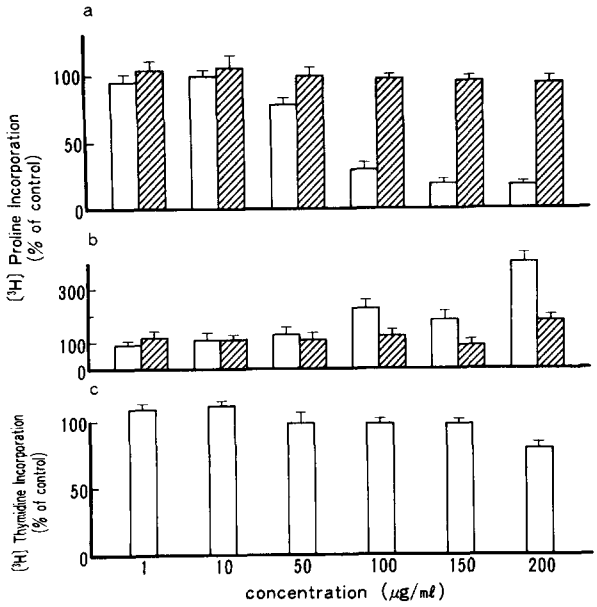

Fig. 1. Effect of an aqueous extract from *S. miltiorrhizae* Radix on collagen (a, b) and DNA (c) synthesis in human skin fibroblasts. Cells treated with extracts for 48 h were labeled with [^{3}H]proline for the final 18 h. The amount of radioactivity incorporated into collagen (open column) and noncollagen proteins (hatched column) was measured after digestion of the medium (a) and cell layer (b) with bacterial collagenase. For measurement of DNA synthesis (c), cells labeled with [^{3}H]thymidine for the final 1 h of the treatment were analyzed for TCA-isoluble radioactivity. Values are mean and SD from duplicate experiments.

3.2. Isolation of inhibitor from S. miltiorrhizae Radix

We proceeded to purify the material which exhibited the inhibitory activity for collagen production by fibroblast cultures. The purification procedure was monitored by collagenase digestion assay of collagen. The aqueous extract of the herb was first resolved on an HPLC column (MCL-gel, CHP-20). The fraction eluting with 50% methanol (fraction B) exhibited the inhibitory activity (data not shown). After chromatography of this fraction on Sephadex LH-20, the inhibitory activity was found associated with the main peak (data not shown). This fraction gave a single peak

Table 1
Effect of Mg lithospermate and an aqueous extract from *Salviae miltiorrhizae* on collagen prolyl and lysyl hydroxylations in cultured fibroblasts

Treatment	Hyp/Pro	Hyl/Lys
Control	0.71	0.23
Aqueous extract	0.40	0.12
Control	0.76	0.23
Mg lithospermate	0.33	0.09

Cells treated for 48 h with the aqueous extract (150 mg/ml) or Mg lithospermate (100 μg/ml) were labeled with [^{3}H]proline and [^{14}C]lysine for the last 24 h.

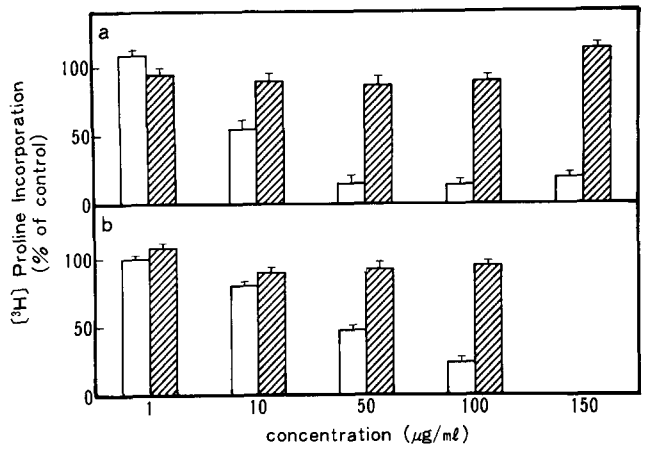

Fig. 2. Effects of fraction B (a) and Mg lithospermate (b) on collagen (open column) and noncollagen protein (hatched column) synthesis in human skin fibroblasts. Cells treated for 48 h were labeled with [^{3}H]proline for the final 18 h. The medium was analyzed by collagenase digestion assay.

when analyzed by HPLC (data not shown). In the course of determining the chemical structure of the inhibitor, it came to our attention that a compound identified as Mg lithospermate had been isolated from S. miltiorrhizae Radix, using a similar purification procedure [10]. We acquired this compound and tested it in our cell culture system. Its identity with the compound in fraction B is suggested from the comparable inhibitory activity for collagen biosynthesis and hydroxylation (Fig. 2, Table 1).

3.3. Comparison of inhibitory activities of fraction B and Mg lithospermate for prolyl and lysyl hydroxylases

Mg lithospermate bears some structural resemblance to a known class of inhibitors of prolyl hydroxylase. Fraction B as well as Mg lithospermate (Fig. 3) at 50 μg/ml were found to inhibit activities of both prolyl and lysyl hydroxylases isolated from fibroblast cultures, but at 5 μg/ml only the activity of lysyl hydroxylase (Table 2). Lysyl hydroxylase activity was also selectively inhibited after treating fibroblast cultures with Mg lithospermate, 250 μg/ml for 48 h or 100 μg/ml for 72 h (Table 3). In a separate experiment with different

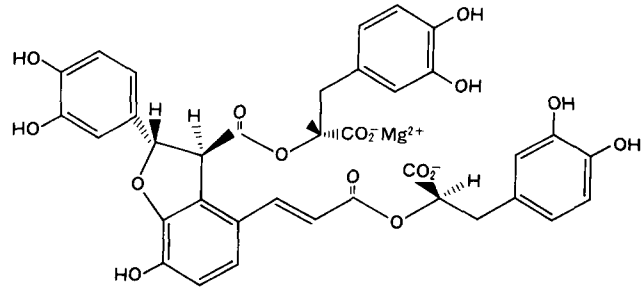

Fig. 3. Chemical structure of magnesium lithospermate.

Table 2
Effect of fraction B and Mg lithospermate on activities of prolyl and lysyl hydroxylases isolated from fibroblast cultures

Treatment	Conc. (μg/ml)	Prolyl hydroxylase activity (% of control)	Lysyl hydroxylase activity (% of control)
Control	0	100	100
Fraction B	5	81	45
	50	6	1
Mg lithospermate	5	91	48
	50	7	0

Cell extracts were preincubated with 5 or 50 μg/ml of fraction B or Mg lithospermate for 45 min at 25°C, then assayed for prolyl and lysyl hydroxylase activities as described in Materials and Methods.

Table 3
Effect of treating fibroblast cultures with Mg lithospermate on prolyl and lysyl hydroxylase activities

Duration	Conc. (μg/ml)	Prolyl hydroxylase activity (% of control)	Lysyl hydroxylase activity (% of control)
48 h	0	100	100
	10	116	110
	50	116	116
	100	113	110
	250	102	63
72 h	0	100	100
	1	104	103
	10	96	93
	100	103	57

Cells were treated for 48 for 72 h with various concentrations of Mg lithospermate as indicated. Prolyl and lysyl hydroxylase activities in cell extracts were determined as described in 'Materials and Methods'.

enzyme preparation, both activities were found to be equally sensitive to inhibition by Mg lithospermate; 50 μm iron completely reversed the inhibition at 5 μg/ml and partially reversed it at 50 μg/ml (Table 4).

3.4. Skin collagen production in mice treated with Mg lithospermate

The recovery of acid soluble collagen from drug-treated mice was higher than control (330 mg vs. 20 mg), as was total radioactivity (hydroxyproline plus proline) per extracted acid soluble collagen (4.1×10^3 cpm vs. 2.6×10^3 cpm). The ratio of hydroxyproline to proline radioactivity recovered chromatographically after hydrolysis of acid soluble collagen was significantly reduced after drug treatment in comparison to control, indicating that Mg lithospermate is capable of inhibiting prolyl hydroxylation in newly synthesized collagen (Table 5)

4. Discussion

S. miltiorrhizae is a well-known traditional medicinal herb widely prescribed in China to improve blood circulation and relieve stasis. Having vasodilatory, hypotensive, anticoagulant, and antibacterial activities, it has also been reported beneficial for patients with chronic renal failure [11]. In fact, its oral administration to rats has been found to increase renal blood flow

Table 4
Iron-dependent inhibition of prolyl and lysyl hydroxylase activities by Mg lithosphermate

Treatment	Prolyl hydroxylase activity		Lysyl hydroxylase activity	
	5 μM Fe^{2+} (% of control)	50 μM Fe^{2+} (% of control)	5 μM Fe^{2+} (% of control)	50 μM Fe^{2+} (% of control)
Control	100	100	100	100
Mg lithospermate, 5 μg/ml	61	94	56	103
Mg lithospermate, 50 μg/ml	14	35	7	41

Cell extracts were preincubated at 25° C with Mg lithospermate (0, 5 or 50 μg/ml) for 45 min, then with 5 or 50 μM $FeSO_4$ for 15 min. Enzyme activities were assayed as described in 'Materials and Methods'.

Table 5
Effect of Mg lithospermate on mouse skin collagen synthesis and hydroxylation

Treatment	Acid soluble collagen (mg/g dry wt. skin)	Acid soluble residue (mg/g dry wt. skin)	Newly synthesized collagen (cpm/mg dry wt. acid soluble collagen)	Hydroxyproline (cpm/mg dry wt. acid soluble collagen)	Proline (cpm/mg dry wt. acid soluble collagen)	Hyp/Pro
Control	220 ± 40	780 ± 40	$2.6 \pm 0.3 \times 10^3$	690 ± 190	1032 ± 330	0.68 ± 0.03
Mg lithospermate	330 ± 30	670 ± 20	$4.1 \pm 0.2 \times 10^3$	934 ± 70	2334 ± 574	0.41 ± 0.08

Mice were treated with Mg lithospermate (1 mg/kg per day) for 30 days and labeled with [^{3}H]proline for 6 h on the last day. Acid soluble collagen was extracted from skin, and the amount of radioactivity incorporated into collagen proline and hydroxyproline was determined as described in 'Materials and Methods'. Values are mean ± S.D. from three mice.

and significantly decrease serum urea nitrogen, creatinine, methyl guanidine, and guanidinosuccinic acid [12–14]. The active component of this herb has been isolated and identified as magnesium lithospermate [10]. Having three catechol moieties, this compound was expected to chelate iron at the α-ketoglutarate or ascorbate binding site of prolyl hydroxylase [15]. Activities of both prolyl and lysyl hydroxylases isolated from fibroblast cultures were inhibited by Mg lithospermate. However, only lysyl hydroxylase activity was inhibited after treating fibroblast cultures with Mg lithospermate, although the collagen synthesized was deficient in both hydroxyproline and hydroxylysine. The in vitro inhibition of both enzymes by the drug could be completely prevented by excess ion, suggesting an iron chelation mechanism which is consistent with the reversible inhibition of prolyl hydroxylase but not the irreversible inhibition of lysyl hydroxylase in the cell; the latter must occur by a different mechanism. Administered to mice, Mg lithospermate was found to significantly reduce the degree of prolyl hydroxylation in newly synthesized skin collagen from 0.68 to 0.41. However, total incorporated radioactivity in collagen extracted from drug-treated mice was higher than control (4.1×10^3 vs 2.6×10^3). This may have been due to increased solubility of collagen from drug-treated mice, as a result of the formation of underhydroxylated collagen. This was reflected in the amount of acid soluble collagen extracted from drug-treated and control mice (330 vs. 220).

We have provided evidence showing that Mg lithospermate inhibits collagen prolyl and lysyl hydroxylations in cultured fibroblasts by inhibiting prolyl and lysyl hydroxylase activities. The accompanying inhibition of procollagen secretion observed in drug-treated cells may be related to the inhibition of prolyl hydroxylation. Certain compounds containing a catechol moiety have been reported to inhibit prolyl hydroxylation during collagen biosynthesis, e.g. ethyl 3,4-dihydroxybenzoate in human skin fibroblasts [16] and 2,7,8-trihydroxyanthraquinone in chick tendon cells [17]. To our knowledge none of these compounds have been tested in experimental animals, making their therapeutic potential uncertain. The ability of Mg lithospermate to inhibit collagen prolyl hydroxylation in vitro as well as in vivo warrants further studies in experimental models of fibrosis. As a natural product with extensive clinical experience in China indicating no overt toxicity, Mg lithospermate promises to be a valuable antifibrotic drug.

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