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Research Paper

Lapachol suppresses cell proliferation and secretion of interleukin-6 and plasminogen activator inhibitor-1 of fibroblasts derived from hypertrophic scars

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Abstract

Objectives The pathogenesis and therapy of hypertrophic scar have not yet been established. Our aim was to investigate the antiproliferative and antisecretory effects of lapachol, isolated from the stem bark of *Avicennia rumphiana* Hall. f., on hypertrophic scar fibroblasts.

Methods The effects of lapachol on hypertrophic scar fibroblast proliferation were measured using the MTT assay, cell-cycle analyses and lactate dehydrogenase assays. The type I collagen α-chain (COL1A1), interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) mRNA and/or protein levels of hypertrophic scar-fibroblasts were quantitated by real-time PCR and ELISA.

Key findings Lapachol at 25 and 50 μM significantly inhibited the *in vitro* proliferation of hypertrophic scar fibroblasts, but not fibroblasts from non-lesional skin sites. In addition, lapachol had no apparent effect on cell cycle and lactate dehydrogenase activity in conditioned medium from lapachol-treated hypertrophic scar fibroblasts was nearly equal to that in medium from vehicle-treated cells. Lapachol treatment also inhibited COL1A1 and PAI-1 mRNA levels in hypertrophic scar fibroblasts, but did not affect IL-6 mRNA levels. The protein levels of IL-6 and PAI-1 in conditioned medium from hypertrophic scar fibroblasts treated with 50 μM lapachol were lower than those from vehicle-treated hypertrophic scar fibroblasts.

Conclusions Lapachol decreased the proliferation rate of hypertrophic scar fibroblasts. As IL-6 and PAI-1 secretion was also lowered in lapachol-treated hypertrophic scar fibroblasts, our findings suggested that lapachol may have suppressed extracellular matrix hyperplasia in wound healing and possibly alleviated the formation of hypertrophic scar.

Keywords lapachol; fibroblast; hypertrophic scar; IL-6; PAI-1

Introduction

Hypertrophic scar (HS) is a common fibroproliferative disorder of the human dermis characterized by erythematous, raised, pruritic lesions of the healing skin that are often itchy and painful. They lead to a reduction in the quality of life. HS typically occurs following thermal and other injuries that involve the deep dermis, and develops due to the excess deposition of collagen. To date, an effective therapy for HS has not been developed.

The key cells involved in HS are dermal fibroblasts, which are known to increase in HS lesion sites where they produce and secrete collagen, cytokines and chemokines. These secreted molecules induce fibroblast proliferation and migration of inflammatory cells, leading to fibrosis.[1,2] A few reports have shown that interleukin (IL)-6 levels in conditioned medium from HS fibroblasts are higher than those in medium from fibroblasts derived from normal sites.[3,4] Ghazizadeh[5] reported that IL-6 accelerated fibroblast proliferation via autocrine action. Although plasminogen activator inhibitor type 1 (PAI-1) is known to control fibrinolysis in blood vessels, recent studies have revealed that PAI-1 also modulates tumour metastasis.[6–8] During fibrosis, the secretion of PAI-1 by fibroblasts may indirectly suppress degradation of the extracellular matrix through the inhibition of urokinase.[9,10] The formation of HS is therefore complicated by several factors.

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Current approaches for the treatment of HS often involve pressure treatment and the administration of antihistamine or steroid drugs; however, these therapies are not fully effective. Recent studies reported that green tea and epigallocatechin-3-gallate inhibited the proliferation of fibroblasts derived from keloids, which is a disease similar to HS.[11,12] Despite their promising therapeutic potential, few studies have examined the effects of natural products on fibroblasts, particularly with respect to HS.

Lapachol, a derivative of naphthoquinone, is a natural product isolated from *Tabebuia avellandedae* and is used in traditional medicine. Although several reports have described the various biological activities of lapachol, including antibacterial, antiparasitic and anticancer effects, the influence of lapachol on fibroblasts is not well understood.[13–16] In this study, we have investigated the pharmacological actions of lapachol on HS fibroblasts by examining the effects of lapachol on cell proliferation, cell cycle and cell death. In addition, we have examined the influence of lapachol on type I collagen α-chain (COL1A1) mRNA expression and the mRNA and protein levels of PAI-1 and IL-6 in HS fibroblasts.

Materials and Methods

Plant material

Avicennia rumphiana Hall. f. (Avicenniaceae) (MUY0061).

Extraction and isolation of lapachol

For the extraction of lapachol, dried twigs (660 g) of *A. rumphiana* collected in Singapore were subjected to extraction with acetone. The resulting acetone extract (4.6 g) was then subjected to silica-gel column chromatography and successive elutions with hexane, hexane–acetone (19 : 1, 18 : 2, 17 : 3, 4 : 1, 7 : 3, 1 : 1, 1 : 2), acetone, CH_2Cl_2–MeOH (3 : 1) and MeOH to give 11 fractions. Fraction 5 (287 mg, hexane–acetone, 4 : 1) was further chromatographed on silica gel and eluted with hexane–acetone (5 : 1) to yield lapachol (12.2 mg). Lapachol was identified by comparison of the ^{1}H NMR and IR data with an authentic sample (Figure 1a).

Lapachol; yellow prism. HRMS *m/z*: calcd for $C_{15}H_{14}O_3$: 242.0934, found: 242.0944. IR μmax ($CHCl_3$): 3406, 1653 cm^{-1}. UV λmax (MeOH): 207, 252, 276, 330, 388 nm. EIMS *m/z*: 242 (M^+, 52%), 228 (30%), 227 (100%), 199 (14%), 181 (9%), 179 (8%), 152 (8%). ^{1}H NMR (400 MHz, $CDCl_3$) 8.12 (1H, dd, *J* 7.7, 1.0 Hz), 8.07 (1H, dd, *J* 7.7, 1.0 Hz), 7.75 (1H, td, *J* 7.7, 1.0 Hz), 7.67 (1H, td, *J* 7.7, 1.0 Hz), 5.21 (1H, m), 3.31 (1H, d, *J* 7.3 Hz), 1.79 (3H, s), 1.69 (3H, s).

The purity of the test compound was confirmed by high-resolution MS, HPLC, and ^{1}H NMR spectra (Figure 1b). Samples were dissolved in dimethyl sulfoxide (DMSO) and were added to culture medium to give a final DMSO concentration of 0.1% v/v, at which concentration DMSO had no significant effect on the growth of the tested cells (data not shown).

Preparation and culture of fibroblasts

Human skin biopsies were obtained from patients after informed consent was given, according to the guidelines established by Aichi Medical University, which follow the principles expressed in the Declaration of Helsinki. Tissue samples were collected from Japanese patients undergoing excision of HS ($n = 3$) or non-lesional skin ($n = 3$) at the Department of Plastic and Reconstructive Surgery, Aichi Medical University, Japan. Samples were obtained from HS lesions and from intact skin as a control.

For the culture of fibroblasts, normal and HS skin samples were minced, placed in sterile 30-mm dishes and lightly pressed with a coverslip. After the addition of 3 ml RPMI-1640 supplemented with 100 U/l benzylpenicillin, 100 µg/l streptomycin, 0.1 mM nonessential amino acids, 2 mM glutamine, 50 mg/l pyruvic acid and 10% fetal calf serum, the dishes were incubated under sterile conditions at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed once a week until confluency (4–6 weeks). The fibroblasts used for experiments were between the second and fourth generation and were seeded (1×10^4 cells/well) in culture slides containing the identical culture medium.

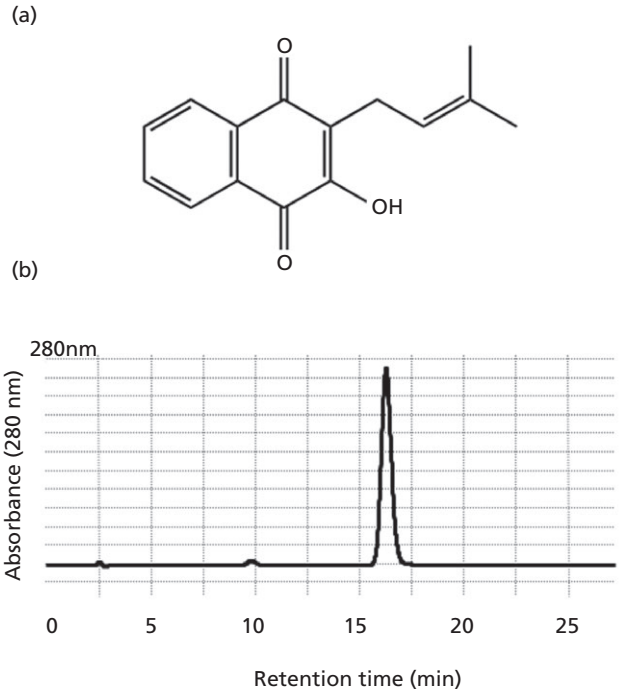

Figure 1 Structure (a) and HPLC analysis (b) of lapachol isolated from *Avicennia rumphiana* Hall. f.

Fibroblast proliferation

Fibroblasts (5×10^3 cells/well) were seeded in 96-well dishes and cultured overnight in RPMI-1640 as described above. The following day (day 0), the medium was exchanged with fresh medium. Cell proliferation was measured by colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) assay at specific concentrations of lapachol and times.

Flow cytometry

Cells were seeded at 3×10^5 cells per 60-mm plate in RPMI-1640 with or without lapachol (25 µM). After culturing the

cells for five days, they were trypsinized from the plates. Cells were fixed in 70% ethanol and treated with ribonuclease (0.5%) for 30 min. DNA was stained with propidium iodide (5 μg/ml) for 30 min at room temperature in the dark. Flow cytometry and cell cycle analysis were performed using a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) with 488-nm excitation. The forward and side scatter properties of the cells were used to establish size gates and exclude cellular debris. For each sample, at least 10 000 gated cells were examined.

Lactate dehydrogenase activity

Cell viability was determined by a lactate dehydrogenase (LDH) assay. LDH activity was measured in conditioned medium from lapachol-treated and vehicle (DSMO)-treated HS fibroblasts using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega Co., Madison, WI, USA) according to the manufacturer's protocol.

Quantitative analysis of COL1A1, PAI-1 and IL-6 mRNA

Human fibroblasts (3×10^4 cells/well) were seeded in 96-well dishes and incubated with lapachol at specific concentrations at 37°C for 3 h. COL1A1, PAI-1 and IL-6 mRNAs were analysed by quantitative real-time PCR using ABI-9500 (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from fibroblasts using an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions and was reverse-transcribed to prepare cDNA using a high-capacity cDNA Archives Kit (Applied Biosystems). Real-time PCR was performed using 20 ng obtained cDNA with SYBR Premix EX Taq (Takara Co., Otsu, Japan). PCR conditions comprised 10 s at 95°C, and 40 cycles at 95°C for 5 s and 60°C for 34 s, followed by standard melting curve analysis. An internal standard curve was generated by amplification of fivefold dilutions of cDNA for each gene during each run. The number of copies in each real-time PCR reaction was normalized against a housekeeping gene (GAPDH). The primers for the real-time PCR assays were designed using the Primer 3 program (http://frodo.wi.mit.edu/primer3) with mRNA coding sequences derived from GenBank.

The following primers were used: COL1A1: forward primer 5′-GTGCTAAAGGTGCCAATGGT-3′ and reverse primer 5′-ACCAGGTTCACCGCTGTTAC-3′; PAI-1: forward primer 5′-CCCTTTGCAGGATGGAACTA-3′ and reverse primer 5′-TGGCAGGCAGTACAAGAGTG-3′; IL-6: forward primer 5′-GCATGATCCGAGATGTGGAA-3′ and reverse primer 5′-ACGAGCGGGAATGAGAAGAG-3′; GAPDH: forward primer 5′-TGCCACTCAGAAGACTG TGG-3′ and reverse primer 5′-GGATGCAGGGATGATGT TCT-3′.

Measurement of PAI-1 and IL-6 levels in conditioned medium

Human fibroblasts (5×10^4 cells/well) were seeded in 24-well dishes and incubated with lapachol at specific concentrations at 37°C for five days. PAI-1 and IL-6 levels in the conditioned medium of lapachol-treated and vehicle-treated fibroblasts were determined using human SerpinE1/PAI-1 and IL-6 ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

Measurement of total protein in hypertrophic scar fibroblasts

After collecting conditioned medium from the five-day cultured lapachol-treated and vehicle-treated fibroblasts for the measurement of PAI-1 and IL-6 concentrations, the remaining cells were briefly lysed in CelLytic M Cell Lysis Reagent (Sigma) according to manufacturer's protocol. Total protein of its cell lysate was measured by a DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Differences in the various treatments were statistically examined using the Kruskal–Wallis test. Individual differences between treatments were examined with the Dunn's-test using the SAS GLM procedure. Data are expressed as the mean ± standard deviation (SD). P-values < 0.05 were considered significant compared with vehicle (DMSO)-treated fibroblasts.

Results

Morphology and cell proliferation of lapachol-treated hypertrophic scar fibroblasts

As determined by phase contrast microscopy, lapachol-treated HS fibroblasts showed no chromatic agglutination or morphological changes, such as swelling or rounding of cells. In addition, after five days of culture, lapachol-treated cells did not become confluent as compared with vehicle-treated cells (Figure 2a).

The rate of proliferation of lapachol-treated HS fibroblasts at 25 or 50 μM was significantly lower than that of vehicle-treated HS fibroblasts (Figure 2b), although treatment with 5 μM lapachol did not affect cell proliferation. The proliferation rate of HS fibroblasts treated with lapachol at 25 or 50 μM were decreased 28.8 and 42.9%, respectively, compared with vehicle-treated HS fibroblasts. However, in fibroblasts from non-lesional skin sites, the proliferation rate after lapachol treatment was not markedly different from vehicle-treated cells (Figure 2b). Furthermore, the growth inhibition of lapachol at 25 μM on HS fibroblasts was not a time-dependent effect (Figure 2c). From these results, it was concluded that lapachol specifically inhibited the proliferation of HS fibroblasts at concentrations of 25 or 50 μM.

The influence of lapachol on cell cycle and lactate dehydrogenase activity of hypertrophic scar fibroblasts

Based on the observed effects of lapachol treatment on cell morphology and proliferation, we investigated the influence of lapachol on cell cycle in HS fibroblasts using FACS analysis (Figure 3). Although lapachol treatment showed a slight tendency to induce an increase in the percentage of cells in the G2/M phase compared with the control fibroblasts, this change was not significant (Figure 3a). The changes in the population of cells in all other phases were also negligible

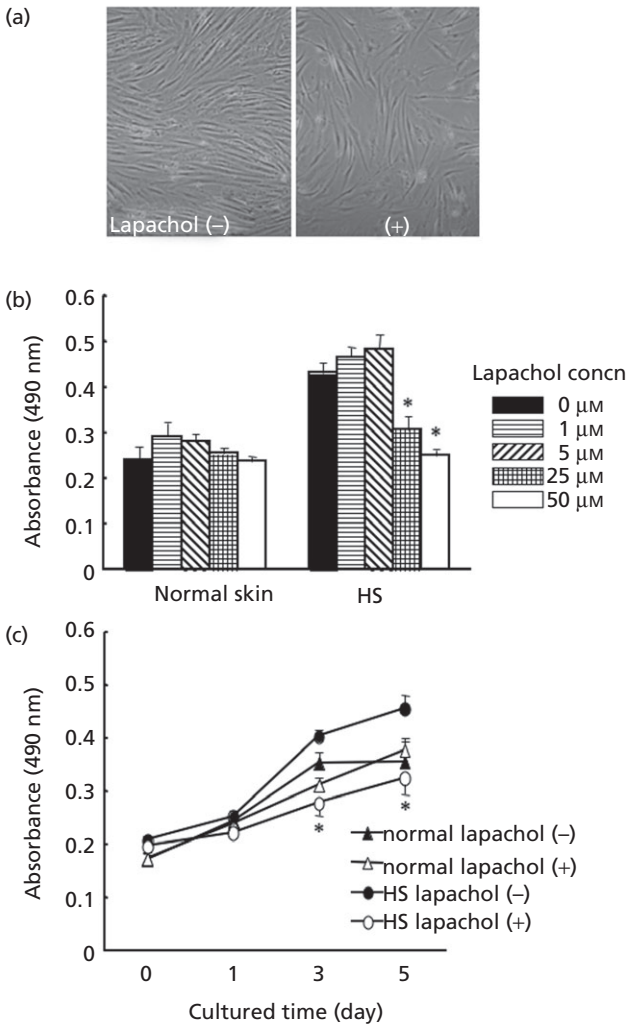

Figure 2 Lapachol inhibited the proliferation of hypertrophic scar-derived fibroblasts. (a) Fibroblasts derived from hypertrophic scar (HS) were observed using phase contrast microscopy after treatment with 25 μM lapachol (right) or vehicle (left) for five days. Original magnification × 60. (b) Fibroblasts derived from HS and normal skin were treated with various concentrations of lapachol (0 μM was the vehicle dimethyl sulfoxide (DMSO)) and cultured for five days. Cell proliferation was assessed using a colorimetric MTT assay. Data are presented as the mean ± SD of three independent experiments. $*P < 0.05$ vs vehicle-treated cells in each group. (c) Fibroblasts were treated with either vehicle (DMSO) or 25 μM lapachol and cultured. Cell proliferation is presented as the means ± SD of three independent experiments. $*P < 0.05$ vs vehicle-treated cells in each group.

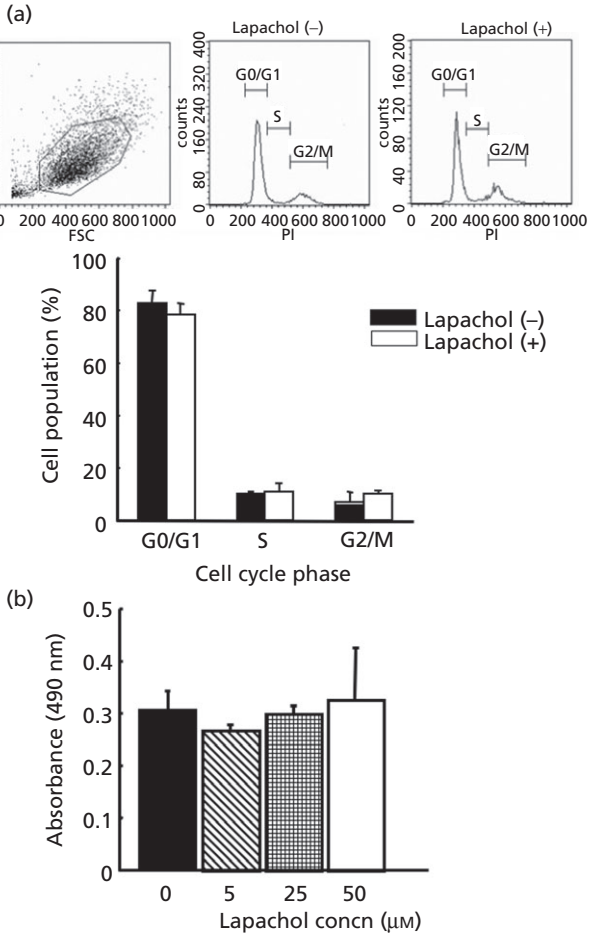

Figure 3 Lapachol did not affect cell cycle or viability of hypertrophic scar fibroblasts. (a) Hypertrophic scar (HS)-derived fibroblasts were treated with either vehicle (dimethyl sulfoxide, black bar) or 25 μM lapachol (white bar) and cultured for five days. The upper panels show the cell cycle analysis of HS fibroblasts. Figure show the patterns of flow cytometry of vehicle-treated HS fibroblasts (right), and the histograms of cell cycle of vehicle-treated (centre) and lapachol-treated (left) HS fibroblasts. Forward scatter (FSC) and side scatter (SSC) reflect cell size and intracellular structures, respectively. Propidium iodide (PI) reflects the levels of DNA. The lower graph shows the percentage of cells in the different phases of the cell cycle. Values are the mean ± SD of three independent experiments. (b) Fibroblasts were treated with various concentrations of lapachol (0 μM was the vehicle dimethyl sulfoxide (DMSO)) and cultured for five days to determine if lapachol induced necrosis, as determined from the lactate dehydrogenase (LDH) activity. LDH activity is presented as the mean ± SD of three independent experiments. $*P < 0.05$ vs vehicle-treated cells in each group.

between the two treatment groups. When LDH activity was examined in conditioned medium from lapachol-treated HS fibroblasts to estimate cell death, it was found to be nearly identical to that of vehicle-treated cells (Figure 3b). Taken together, these results demonstrated that lapachol had little effect on cell cycle and cell death of HS fibroblasts.

Effect of lapachol on COL1A1, PAI-1 and IL-6 mRNA levels in hypertrophic scar fibroblasts

To investigate the effects of lapachol on the production of inflammatory molecules by HS fibroblasts, the mRNA levels of COL1A1, PAI-1 and IL-6 were measured by real-time PCR in HS fibroblasts treated with lapachol 0–50 μM (Figure 4). The levels of PAI-1 and COL1A1 mRNA in lapachol-treated HS fibroblasts were significantly reduced compared with the levels in vehicle-treated cells (Figure 4a and b). Notably, the PAI-1 and COL1A1 mRNA levels did not decrease in a dose-dependent manner. For IL-6, the mRNA levels in lapachol-treated HS fibroblasts were equal to those of vehicle-treated cells (Figure 4c) showing that lapachol had no effect on the transcription of IL-6 at these concentrations.

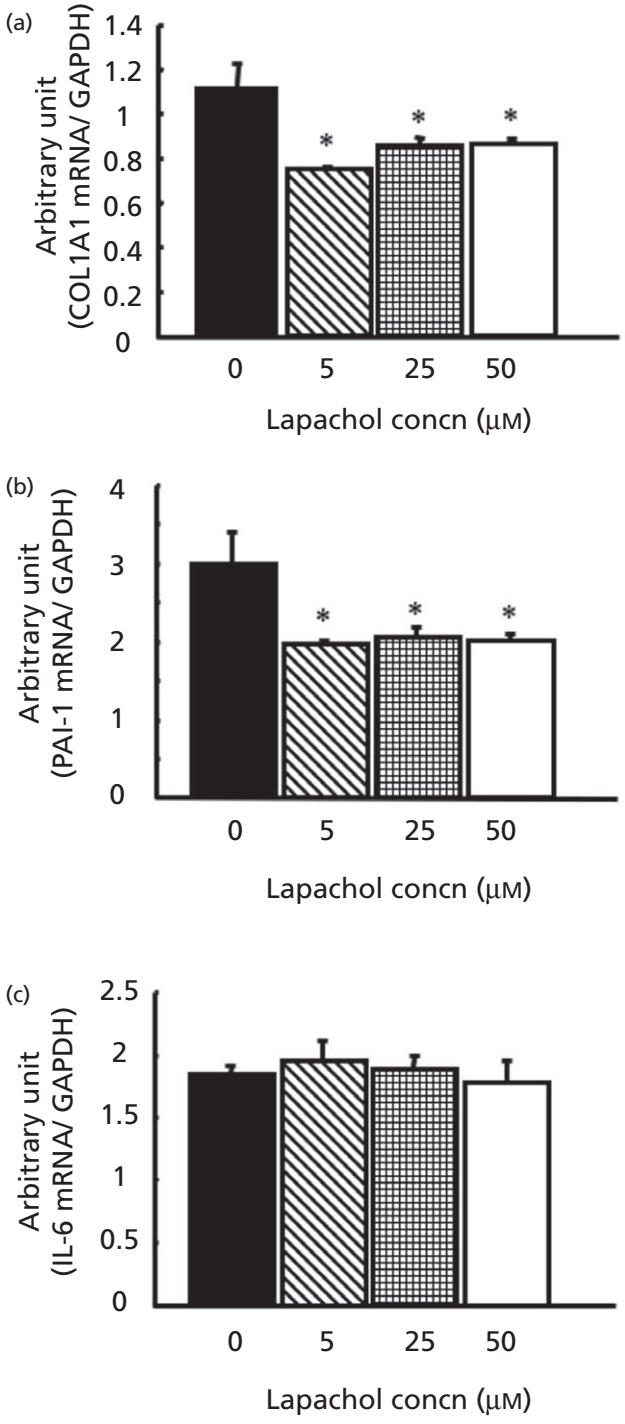

Figure 4 Lapachol reduced COL1A1 and PAI-1 mRNA levels in hypertrophic scar-derived fibroblasts. Fibroblasts were treated with various concentrations of lapachol (0 μM was the vehicle dimethyl sulfoxide (DMSO)) and cultured for 3 h. The levels of COL1A1 (a), PAI-1 (b) and interleukin (IL)-6 (c) mRNA in hypertrophic scar (HS)-derived fibroblasts were measured by real-time PCR. The arbitrary units (mRNA levels) are presented as the mean ± SD of three independent experiments. $*P < 0.05$ vs vehicle-treated cells in each group.

Effect of lapachol on PAI-1 and IL-6 protein levels in hypertrophic scar fibroblasts

Finally, the total proteins in HS fibroblasts and the levels of PAI-1 and IL-6 protein in conditioned medium from HS fibroblasts were measured using DC Protein Assay and ELISA kits (Figure 5). At all examined concentrations, lapachol treatment did not affect the total protein levels of HS fibroblasts (Figure 5a). At 5 μM, lapachol treatment had little effect on the PAI-1 and IL-6 protein levels in HS fibroblasts; however, at 25 μM the PAI-1 and IL-6 protein levels tended to decrease, as compared with vehicle-treated cells. When the concentration was increased to 50 μM, lapachol significantly decreased PAI-1 and IL-6 protein levels in conditioned medium from HS fibroblasts compared with the levels in medium from vehicle-treated cells (Figure 5b and c).

Discussion

Naturally occurring nathoquinones, such as lapachol, display a variety of biological activity that includes analgesic, anti-inflammatory, antineoplastic and diuretic actions.[17] However, as the biological activity of lapachol on fibroblasts was not well understood, we examined the effects of lapachol on cell proliferation and cytokine production in HS fibroblasts. When added to cell cultures at concentrations of 25 or 50 μM, lapachol decreased the cell numbers of HS fibroblasts, but not fibroblasts derived from non-lesional skin sites. In addition, although treated cells could be propagated, the growth rate of lapachol-treated HS fibroblasts was lower than that of vehicle-treated cells.

Fibroproliferative diseases such as HS and keloid are known to involve the overgrowth of fibroblasts.[3,18] We recently reported that higher levels of [^{3}H]thymidine uptake and increased populations of S- and G2/M phase-cells were observed in cultured fibroblasts derived from keloid lesions as compared with those from non-lesional skin sites.[19]As the proliferation rates and cell populations of HS fibroblasts identified in this study resembled those of fibroblasts derived from keloids, HS fibroblasts also appeared to have high growth potential. Based on the reduced cell numbers and proliferation of lapachol-treated HS fibroblasts, we examined the effects of lapachol on cell cycle and cell death. From the results of FACS analysis and LDH activity, lapachol treatment did not appear to have an effect on the cell cycle or the induction of cell death. These results indicated that lapachol limited the overall proliferation rate of HS fibroblasts, although the mechanism is not yet clear. This speculation was supported by several reports that have demonstrated that lapachol suppressed the proliferation of a number of tumour cell lines.[20–22]

The typical fate of HS is the deposition of excessive extracellular matrix, which is a result of fibrosis. HS fibroblasts are known to excessively produce many types of extracellular matrix materials, such as type I collagen, in addition to the production and secretion of various inflammatory molecules.[23] In this study, lapachol treatment decreased both collagen (COL1A1) and PAI-1 mRNA levels in HS fibroblasts in a non-dose-dependent manner, but did not affect IL-6 mRNA levels. These results indicated that lapachol partially inhibited the transcription of collagen and PAI-1. Although the effects of lapachol on the kinetics of gene expression in

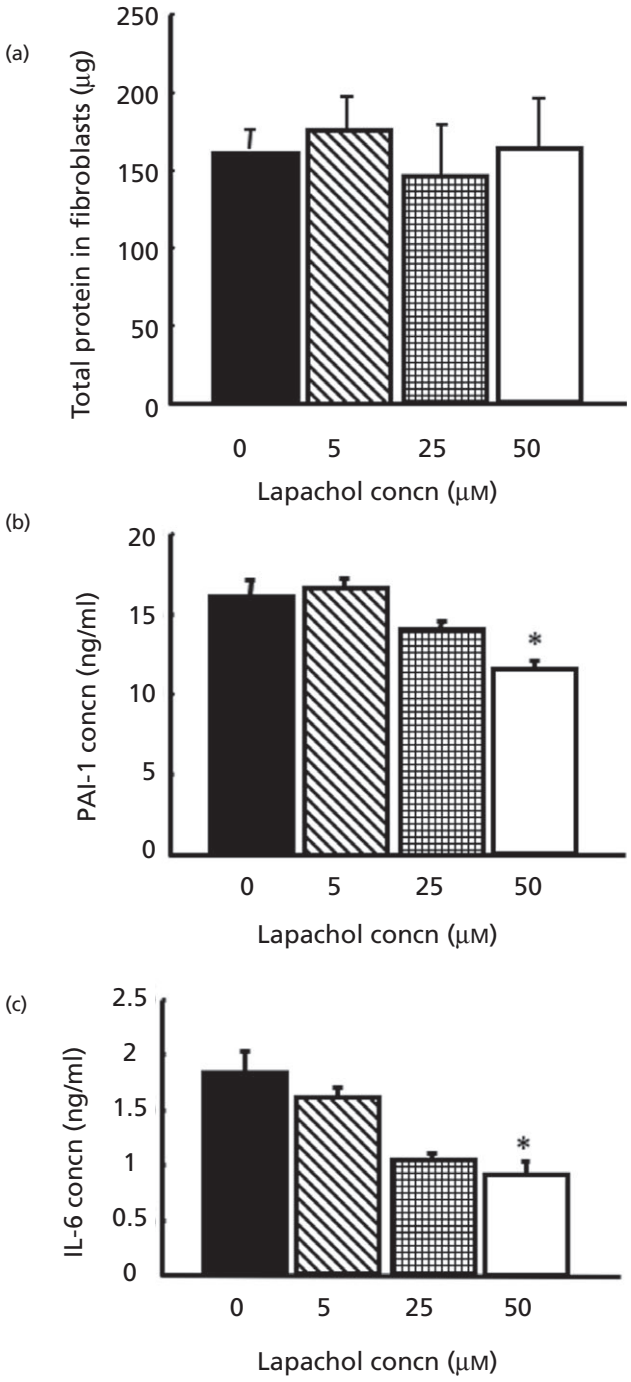

Figure 5 Lapachol inhibited PAI-1 and interleukin-6 secretion from hypertrophic scar-derived fibroblasts. Fibroblasts were treated with various concentrations of lapachol (0 μM was the vehicle dimethyl sulfoxide (DMSO)) and cultured for five days. Total protein levels (a) in vehicle- and lapachol-treated hypertrophic scar (HS)-derived fibroblasts were measured. The protein levels of PAI-1 (b) and interleukin (IL)-6 (c) in conditioned medium from vehicle- and lapachol-treated HS-derived fibroblasts were determined. Protein levels are presented as the mean ± SD of three independent experiments. $*P < 0.05$ vs vehicle-treated cells in each group.

HS fibroblasts cannot be ignored, the inhibition of PAI-1 mRNA levels led to reduced protein secretion in HS fibroblasts. Interestingly, IL-6 protein levels in conditioned medium of HS fibroblasts also decreased despite the fact that lapachol did not inhibit IL-6 mRNA levels. Although the inhibition mechanism of lapachol is unknown, this result indicated that lapachol appeared to inhibit the translation or secretion stages of IL-6 in HS fibroblasts.

Previous studies reported that the expression of PAI-1 was up-regulated in keloid-derived fibroblasts, suggesting that PAI-1 inhibited degradation of the extracellular matrix leading to fibrosis.[24,25] Xue *et al*.[26] reported that fibroblasts from HS and keloids secreted higher levels of IL-6 than cells derived from normal skin sites. IL-6 is known to affect neutrophil recruitment, keratinocyte proliferation and re-epithelialization, and has been shown to promote fibroblast proliferation via autocrine pathways.[27,28] Hence, it is considered that PAI-1 and IL-6 play an important role in scar formation. As lapachol was observed to reduce the mRNA and protein levels of COL1A1, PAI-1 and IL-6 in HS fibroblasts, it is anticipated that lapachol could partially inhibit scar formation *in vivo*.

The inhibition mechanism of lapachol in HS fibroblasts requires investigation. Based on previous reports, although it is expected that lapachol mediates its effects through signal molecules, our preliminary results (unpublished observations) suggest that lapachol does not affect the expression of STAT3, p38 MAPK or NFκB (data not shown).[5,29] Identification of the inhibitory mechanisms of lapachol will hopefully lead to more effective approaches for the treatment of HS.

Conclusions

This is the first study to report that lapachol suppressed the proliferation of HS fibroblasts. As PAI-1 and IL-6 secretion and production were also lowered in lapachol-treated HS fibroblasts, our findings suggested that lapachol may help prevent the formation of hypertrophic scars.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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