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# Gallic acid inhibits fibroblast growth and migration in keloids through the AKT/ERK signaling pathway

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## Abstract

Keloids are a fibroproliferative disorder of the skin resulted from abnormal healing of injured or irritated skin and are characterized by the ability to spread beyond the original boundary of the wound. Here, we tested the effect of gallic acid (GA), a plant polyphenol with selective growth inhibitory effects in cancer, on the proliferation and invasion of keloid fibroblasts (KFs) isolated from patients undergoing surgery. GA inhibited KF proliferation, migration, and invasion in parallel with the downregulation of matrix metalloproteinase-1 and -3 and upregulation of tissue inhibitors of metalloproteinase-1. Flow cytometric analysis showed that GA inhibited cell cycle progression and induced apoptosis. The effects of GA on KFs occurred in parallel with the inhibition of AKT and ERK1/2, suggesting that GA acts by suppressing the AKT/ERK signaling pathway. In *ex vivo* explant cultures of keloid tissues, GA inhibited the migration of KFs to the wound area and suppressed the expression of angiogenic markers concomitant with the inhibition of collagen deposition. These results identify GA as a potential therapeutic agent for the treatment of keloids and suggest a potential mechanism underlying its protective effect.

**Key words:** keloid fibroblasts, gallic acid, proliferation, migration, matrix metalloproteinase

## Introduction

Keloids are fibroproliferative scars that occur as a consequence of a dysregulated cutaneous wound healing process [1]. They are characterized by the abnormal proliferation of fibroblasts and excessive deposition of collagen extending beyond the borders of a wound [2]. The excessive deposition of thick, hyalinized collagen bundles in keloids is associated with an imbalance between the production and degradation of extracellular matrix (ECM) components [3]. Keloids can form after burns or other skin injuries and they rarely regress. The mechanisms underlying the formation and progression of keloids remain unclear. However, keloid fibroblasts (KFs) are characterized by the overproduction of type I procollagen and high levels of expression of growth factors including vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1 and -β2, and platelet-derived growth factor [4]. In addition, KFs have lower rates of apoptosis and downregulation of apoptosis-related genes [5].

Keloids are treated with a combination of surgical removal, intralesional steroids, oral antihistamines, cryotherapy, laser removal, radiotherapy, occlusive dressings, and immunomodulators [6]. The aim of surgical excision is to promote healing and requires the complete removal of keloid tissues [7]. However, surgical treatment can result in recurrence, and newly formed keloids are often

bigger than the original lesion. Radiation is commonly used postoperatively and has shown significant efficacy in reducing recurrence; however, it is associated with an increased risk of skin cancer. Other treatments such as laser therapy, cryotherapy, and occlusive dressings show limited efficacy and are associated with significant adverse effects [8].

Gallic acid (GA) is a plant polyphenol that is found abundantly in tea, grapes, berries, and wine [9]. GA has antioxidant, antihyperglycemic, antihyperlipidemic, anti-inflammatory, and neuroprotective effects [10,11]. GA has attracted attention for its selective cytotoxic effects against cancer cells, and its antitumorigenic effects were shown in animal models and *in vitro* in tumor cell lines [12,13]. GA has antifungal effects and a protective effect against type 2 diabetes [14,15]. Phan *et al.* [16] reported the role of GA as an inhibitor of keloid growth; however, the underlying mechanisms remain unknown.

In the present study, we examined the effect of GA on KF proliferation, migration, and apoptosis *in vitro* and on keloid growth in *ex vivo* tissues and investigated the underlying mechanisms.

## Materials and Methods

### Human subjects

Surgically excised keloids ($n = 15$) larger than 3 cm × 3 cm × 3 cm were obtained from patients undergoing plastic surgery who did not receive treatment for scarring (**Table 1**). Human tissues were handled under the approval from the Ethics Committee of Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Detailed information on the patients, location of lesions, and harvested areas are provided in **Table 1**.

### Primary cell isolation and *in vitro* culture

The procedure used for the isolation of KFs from surgically excised keloids was described previously [17]. After the removal of the epidermis and subcutaneous connective tissues, specimens were cut into 1–3 $mm^3$ pieces and digested in 0.15% collagenase (Roche Applied Science, Indianapolis, USA) in DMEM (Gibco, Gaithersburg, USA) at 37°C on a shaker for 3 h. The suspension was then filtered through a nylon mesh, and the resulting filtrate was centrifuged at 240 *g* for 5 min. The pellets containing fibroblasts were resuspended and cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) in 10-cm dishes in an incubator at 37°C and 5% $CO_2$. Cells were passaged as necessary and subcultured at a ratio of 1:3. The first passage cells were harvested and analyzed.

**Table 1. Demographic data of keloid samples used in this study**

| Case | Gender | Age (years) | Origin |
|---|---|---|---|
| 1 | F | 27 | Shoulder |
| 2 | F | 40 | Abdomen |
| 3 | M | 20 | Chest |
| 4 | M | 24 | Arm |
| 5 | F | 25 | Shoulder |
| 6 | F | 35 | Perineum |
| 7 | M | 50 | Chest |
| 8 | F | 20 | Chest |
| 9 | M | 28 | Shoulder |
| 10 | M | 26 | Back |
| 11 | M | 32 | Chest |
| 12 | M | 35 | Arm |
| 13 | F | 38 | Perineum |
| 14 | M | 25 | Chest |
| 15 | F | 27 | Chest |

### Scratch wound healing assay

The scratch wound healing assay was performed as described previously with modifications [18]. Briefly, KFs treated with the indicated concentrations of GA (Sigma, St Louis, USA) were seeded into six-well plates coated with 3.85 mg/ml of rat tail collagen (BD Biosciences, San Diego, USA). A scratch was made on the surface of the plate with a pipette tip, and after 48 h, cell migration was assessed with a microscope (Carl Zeiss, Jena, Germany). The number of migrated cells was counted and expressed as a percent of the control.

### Flow cytometric analysis

Dead cell apoptosis kit with Annexin V and propidium iodide (PI) were obtained from eBioscience (San Diego, USA) and used according to the manufacturer's instructions. Multiple-color flow cytometric analysis was performed using FACS Aria (BD Biosciences).

### Cell cycle analysis

Cultured KFs were washed, fixed in chilled 70% ethanol at 4°C for 30 min, and stained with PI (100 μg/ml) before treatment with 1 mg/ml RNase-A (Sigma) for 30 min at 37°C. The DNA content of 10,000 cells was determined with a FACScan flow cytometer (BD Biosciences). Modfit LT 3.0 software (Verity Software House, Topsham, USA) was used for data analysis.

### Quantitative real-time PCR

Total RNA was extracted from cultured cells treated as indicated and reverse-transcribed using the AMV Reverse Transcription System according to the manufacturer's instructions (Takara, Shiga, Japan). For quantitative real-time PCR (qRT-PCR), SYBR Green PCR mix was used on an ABI Prism 7900HT (Applied Biosystems, Foster City, USA). The following conditions were used: incubation for 2 min at 50°C, then 95°C for 10 min, followed by a two-step PCR program including 95°C for 15 s and 60°C for 60 s for 40 cycles. β-actin was used as an internal control. The primers used are listed in **Table 2**.

**Table 2. Sequence of primers used in qRT-PCR**

| Gene | Sequence |
|---|---|
| *MMP-1* forward | 5′-GCAGCTGTAGATGTCCTTGGGGT-3′ |
| *MMP-1* reverse | 5′-CCTCGGTGTTGTAAGGTGGA-3′ |
| *MMP-3* forward | 5′-AGGACAAAGCAGGATCACAGTTG-3′ |
| *MMP-3* reverse | 5′-CCTGGTACCCACGGAACCT-3′ |
| *TIMP-1* forward | 5′-ACTTCCACAGGTCCCACAAC-3′ |
| *TIMP-1* reverse | 5′-GCATTCCTCACAGCCAACAG-3′ |
| *VEGF* forward | 5′-TGCTTCTGAGTTGCCCAGGA-3′ |
| *VEGF* reverse | 5′-TGGTTTCAATGGTGTGAGGACATAG-3′ |
| *VEGFR* forward | 5′-CCTTGAACACAGCTCAAGCA-3′ |
| *VEGFR* reverse | 5′-CCCAGATTATGCGTTTTCCA-3′ |
| *β-actin* forward | 5′-ATCATGTTTGAGACCTTCAA-3′ |
| *β-actin* reverse | 5′-CATCTCTTGCTCGAAGTCCA-3′ |

### Western blot analysis

Proteins were extracted from KFs in lysis buffer and aliquots containing 50 μg of total protein were separated by 10% SDS-PAGE, followed by transferring to polyvinylidene difluoride membranes (Millipore, Bedford, USA). After being blocked with 5% non-fat dried milk in 0.1% TBS with Tween 20 for 2 h at room temperature, membranes were exposed to the following primary antibodies: anti-matrix metalloproteinase (MMP)-1, anti-MMP-3, anti-tissue inhibitors of metalloproteinase (TIMP)-1, anti-Akt, anti-p-Akt, anti-ERK1/2, and anti-p-ERK1/2 (Abcam, Cambridge, UK) antibodies. GAPDH was used as the loading control. Primary antibody incubation was performed overnight at 4°C, followed by incubation with the corresponding horseradish peroxidase conjugated secondary antibodies for 1 h at 37°C. Bands were detected using FluorChem Esystem (Alpha Innotech Corp, Santa Clara, USA).

### Cell proliferation assay

Cell proliferation was assessed using the CCK-8 assay following the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan). Briefly, KFs treated as indicated were seeded into 96-well plates at a density of 5000 cells per well. At the indicated time points, 10 μl of the kit reagent was added to each well, incubated for 2 h, and scanned at 450 nm with a microplate reader (Thermo Scientific, Waltham, USA).

### Matrigel invasion assay

The assays of cell invasion were conducted with a 24-well transwell chamber with a pore size of 8 μm (Costar, New York, USA). Inserts were coated with 30 μl of Matrigel. After different treatments, KFs ($5 \times 10^3$ cells) were seeded into the upper Matrigel chamber containing 100 ml of serum-free medium and incubated for 24 h. Then, DMEM was added into the lower chamber as the chemoattractant. After fixing and staining with 4,6-diamidino-2-phenylindole, the numbers of invaded cells were pictured and counted in five randomly selected fields (×100 fold) under a microscope. All the experiments were repeated in triplicate.

### *Ex vivo* explant culture of human keloid tissues

*Ex vivo* explant culture of keloid tissues was performed as described previously [19]. Briefly, keloid tissues were harvested under aseptic conditions, the epidermis was removed, and the dermis was cut into 3 mm × 2 mm × 2 mm small pieces using a scalpel. The keloid dermal fragments were divided into three groups and seeded into 10-cm culture dishes and cultured for 3 days in 6 ml of endothelial cell medium (Catalog #1001; ScienCell Research Laboratories, Carlsbad, USA) consisting of basal medium, fetal bovine serum, endothelial cell growth supplement, and penicillin/streptomycin solution. After tissue attachment, the medium was substituted with fresh medium with or without GA (100 μM). Representative micrographs were obtained from the location at which the KFs were scratched at the different time points on Days 0 and 7, and the migrated KFs were counted using a hematocytometer.

### Histological and immunohistochemical analysis

Histological and immunohistochemical analyses were performed as described previously [20]. Briefly, keloid pieces were incubated for 3, 6, and 8 days and keloid specimens were collected. The keloid specimens were fixed with 4% paraformaldehyde at 4°C overnight, embedded in paraffin blocks, and sectioned to 5-μm thicknesses. The sections were stained with hematoxylin and eosin (H&E) for routine examination. The thickness of the dermis and of individual collagen bundles was measured in five random fields in each sample. Data are shown in arbitrary units.

Keloid sections were incubated with antibodies against CD31 (Abcam) at a dilution of 1:500. The bound antibodies were visualized using 3,3′-diaminobenzidine as a chromogen (Dako, Carpinteria, USA), and the slides were counterstained with hematoxylin. The numbers of $CD31^+$ vessels were counted in three randomly selected fields under the microscope.

### Statistical analysis

All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, USA). Differences between two groups were calculated using Student's *t*-tests. ANOVA (analysis of variance) was applied for comparison of the means of multiple groups, in which the Student–Newman–Keuls test was further used for the comparison of two groups. $P < 0.05$ was defined as significant. Data were presented as the mean ± SEM.

## Results

### GA inhibits KF proliferation

Based on the literature evidence, we tested the effect of different concentrations of GA on the proliferation of KFs isolated from patients undergoing surgery for keloids. **Figure 1A** shows the molecular formula of GA. The results of the CCK-8 assay showed that GA inhibited KF proliferation, with a statistically significant inhibition observed at 50 and 100 μM (**Fig. 1B**). However, GA treatment at a higher concentration (200 μM) led to toxicity to KFs. Therefore, 100 μM of GA was used as optimal concentration in the subsequent experiments.

### GA inhibits KF migration and invasion

Wound healing experiments were performed to determine the effect of GA on the migration of KFs. The results showed that GA significantly inhibited the migration of KFs in a dose-dependent manner (**Fig. 2A**). In addition, GA inhibited the invasion of KFs in a dose-dependent manner, with a 75% inhibition of invasion at a dose of 100 μM (**Fig. 2B**). The expression of migration ralated factors MMPs and TIMPs was assessed by qRT-PCR and western blot analysis. The results showed that GA significantly inhibited the expression of MMP-1 and MMP-3 but increased TIMP-1 at mRNA and

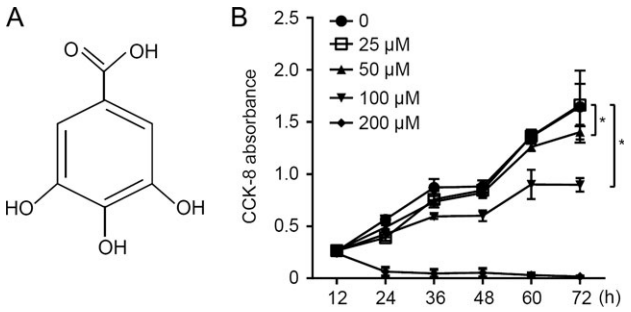


**Figure 1. GA inhibits KF proliferation** (A) Molecular formula of GA. (B) KFs were incubated with different concentrations (25, 50, 100, and 200 μM) of GA for 12–72 h, and cell proliferation was assessed using the CCK-8 assay. Data are shown as the mean ± SEM from three independent experiments ($n = 6$). *$P < 0.05$.

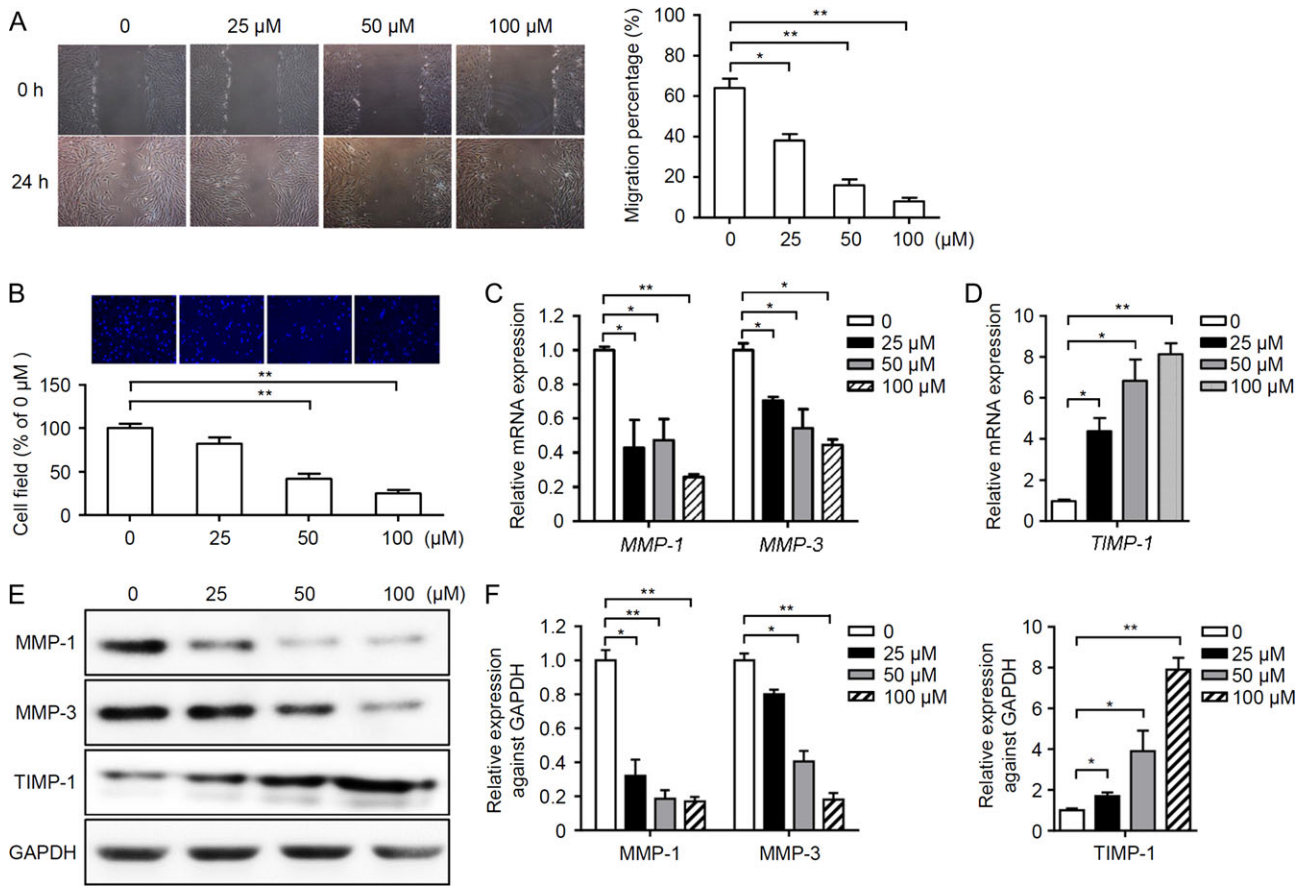


**Figure 2. GA inhibits KF migration and invasion** KFs were treated with different concentrations of GA (0, 25, 50, and 100 μM) and (A) migration was assessed after 24 h using scratch/wound healing assays. Representative images are shown and summarized results were expressed as the mean ± SEM from three independent experiments ($n$ = 6). (B) Cell invasion was assessed after 24 h and representative images (top) are shown and summarized results (bottom) were expressed as the mean ± SEM from three independent experiments ($n$ = 6). (C) The mRNA expression of *MMP-1* and *MMP-3* was assessed by qRT-PCR ($n$ = 6). (D) The mRNA expression of *TIMP-1* was assessed by qRT-PCR ($n$ = 6). (E,F) The protein levels of MMP-1, MMP-3, and TIMP-1 were assessed by western blot analysis. Representative images are shown on the left (E) and summarized results are shown as the mean ± SEM from three independent experiments on the right (F; $n$ = 6). $^*P < 0.05$, $^{**}P < 0.01$.

protein levels (**Fig. 2C–F**). Taken together, our results suggest that GA inhibits KF migration and invasion.

## GA inhibits cell cycle progression and promotes apoptosis in KFs

Cell cycle progression analysis showed that GA increased the number of cells in G1 phase with a concomitant decrease in cells in the S phase of the cell cycle in a dose-dependent manner, indicating that GA caused G1 cell cycle arrest in KFs (**Fig. 3A**). Annexin V/PI staining and flow cytometric analysis showed that GA significantly increased the percentage of apoptotic KFs in a dose-dependent manner, indicating that GA promotes apoptosis in KFs (**Fig. 3B**).

## GA inhibits the AKT/ERK signaling pathway

To determine the mechanism underlying the effect of GA on KF proliferation, the activity of the AKT/ERK signaling pathways was examined by western blot analysis. The results showed that GA downregulated the phosphorylated forms of AKT and ERK1/2 in a dose-dependent manner (**Fig. 4**). Densitometric quantification of bands showed that GA at 100 μM caused 90% downregulation of the relative expression of p-AKT and an ~80% downregulation of p-ERK1/2 in KFs. Taken together, these results indicate that the effects of GA on KFs may be mediated by the AKT/ERK signaling pathway.

## GA inhibits keloid growth and suppresses the expression of angiogenic markers in keloid explant cultures

To confirm the effect of GA on keloid tissues, cells were isolated from *ex vivo* cultures of keloid tissues and treated with different concentrations of GA. *Ex vivo* explant culture experiments showed that GA at 100 μM significantly inhibited the migration of KFs to the wound site (**Fig. 5A**). H&E staining of tissue sections showed that GA inhibited collagen accumulation in keloid explants (**Fig. 5B**). Immunohistochemical staining for CD31 and quantification of the results showed that GA significantly inhibited CD31 positive areas in keloid explants (**Fig. 5C**). In addition, the expression of other angiogenic markers such as VEGF and VEGF receptor (VEGFR) was decreased with GA treatment (**Fig. 5D**), indicating that GA suppressed the expressions of angiogenic markers in keloid tissues.

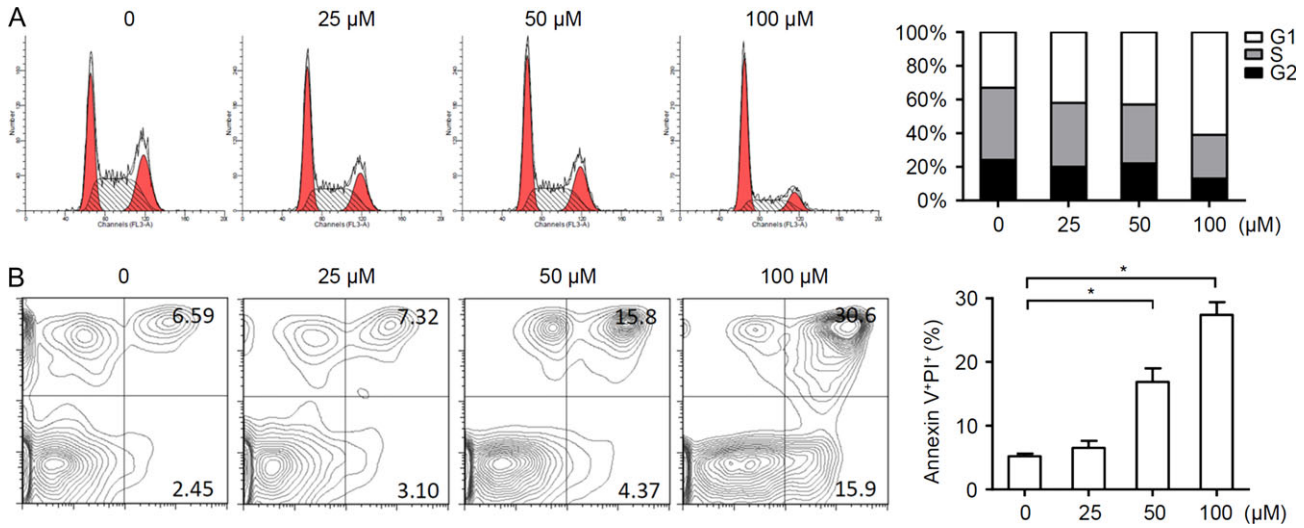


**Figure 3. GA causes G1 cell cycle arrest and promotes KF apoptosis** KFs were treated with different concentrations of GA (0, 25, 50, and 100 μM). (A) Cell cycle progression was analyzed by flow cytometry. (B) Apoptosis was assessed by Annexin V/PI staining. Representative images are shown on the left and summarized results are shown as the mean ± SEM from three independent experiments ($n = 6$). $^*P < 0.05$.

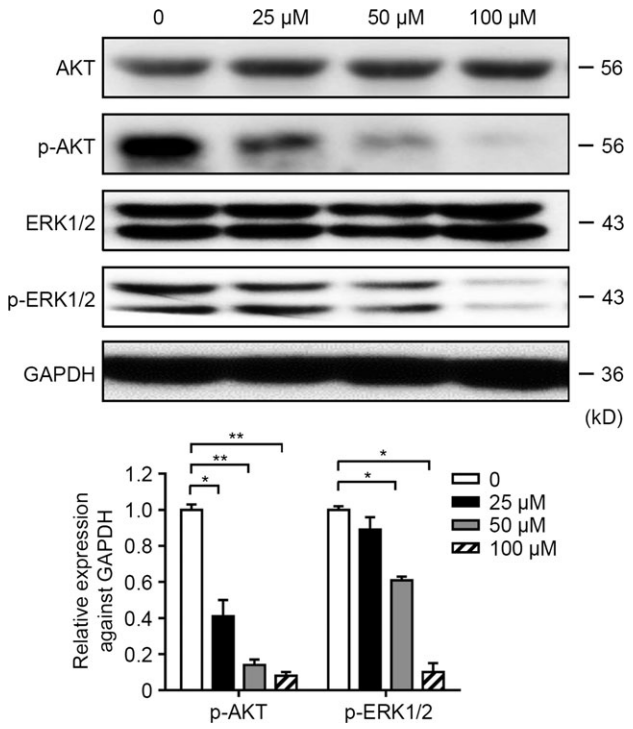


**Figure 4. GA inhibits the AKT/ERK signaling pathway** KFs were treated with different concentrations of GA (0, 25, 50, and 100 μM) and cell lysates were analyzed by western blot analysis against AKT and ERK1/2 and their respective phosphorylated forms. Representative images are shown. Bands were quantified and the results were expressed as the mean ± SEM ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$.

## Discussion

Keloids, which are defined as locally aggressive benign tumors extending beyond the confines of the original wound and invading surrounding skin, are the result of a pathological wound healing process [21]. Despite their nature as benign dermal tumors, keloids do not regress over time and they are characterized by a high rate of recurrence. Furthermore, there are currently no effective curative therapies for keloids despite extensive research and several treatment options [22,23]. In addition, collagen synthesis occurs at rates up to 20 times higher in keloids than in normal skin, and surgical removal of keloids can stimulate collagen deposition leading to the formation of scars larger than the original wound [24]. In the present study, we showed that GA, a naturally occurring triphenolic compound with known antioxidant and apoptosis inducing effects, inhibited KF proliferation, migration, invasion, and cell cycle progression and promoted KF apoptosis, and these effects were mediated by the inhibition of AKT/ERK signaling.

GA has received attention for its numerous pharmacological effects and its potential application in the treatment of many diseases. GA inhibits the proliferation of cancer cells by promoting the generation of reactive oxygen species and inducing cell cycle arrest in G2/M phase [25]. GA was shown to inhibit gastric cancer metastasis by upregulating RhoB and downregulating the AKT/small GTPase signaling, and by inhibiting the NF-κB activity [26]. We showed that GA induced cell cycle arrest at G1 phase and promoted apoptosis in KFs. Further analysis showed that GA inhibited AKT and ERK1/2 activity in KFs in a dose-dependent manner, suggesting that the effect of GA on KFs is mediated by the inhibition of the AKT/ERK signaling. Our findings are therefore consistent with previous studies indicating that the effect of GA may be mediated by the inhibition of the AKT/ERK pathway. Interestingly, there is contradictory data reported by Yang *et al.* [27] demonstrating that GA is beneficial to wound healing by promoting the migration of keratinocytes and fibroblasts under normal and hyperglucidic conditions. In the present study, however, we found that GA suppresses the migration and invasion of fibroblasts derived from keloids. The difference between the roles of GA may result from the origin and characteristics of fibroblasts under different conditions. The mechanisms remain unknown and need to be clarified by further study.

The excessive proliferation of KFs was previously shown to be related to ERK and phosphatidylinositol 3-kinase (PI3K) pathway activation [28]. Keloids are characterized by excessive collagen-ECM deposition and accumulation, and collagen secretion in

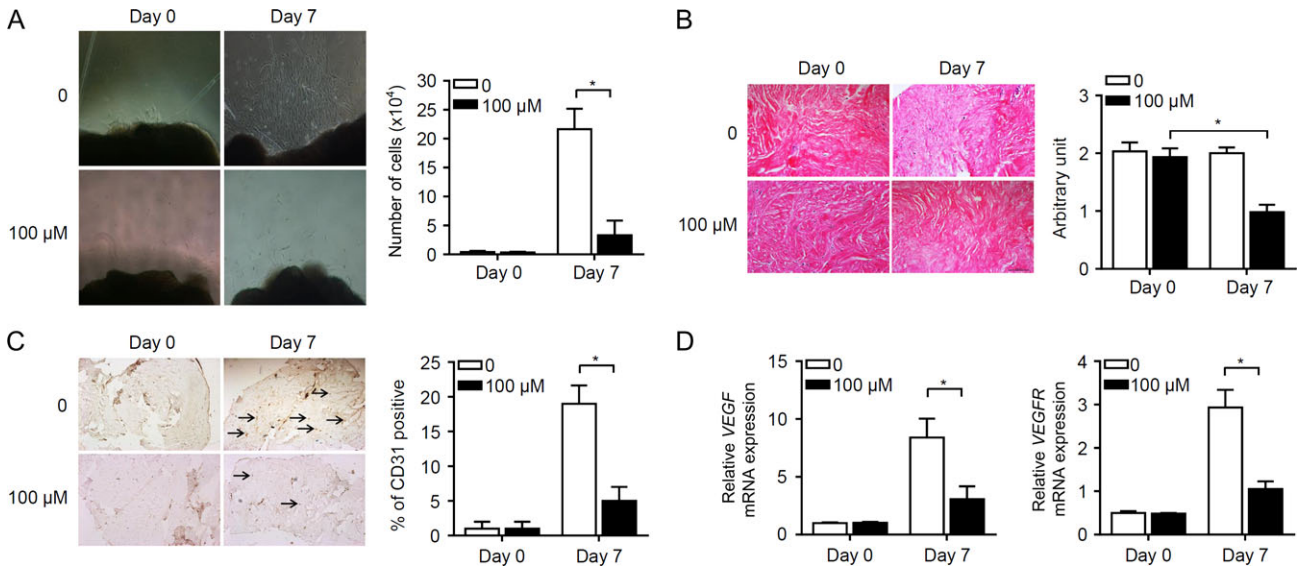


**Figure 5. GA inhibits keloid growth and suppresses the expression of angiogenic markers in keloid explant cultures** (A) KFs were isolated from *ex vivo* explant cultures and treated with or without 100 μM GA. A scratch was generated in cultured KFs and the migration of cells was visualized and quantified ($n = 6$). (B) Representative images of H&E staining of keloid explant tissues treated with or without 100 μM GA are shown on the left. Original magnification, × 100. Graphical presentation of dermal thickness was shown on the right ($n = 6$). (C) Immunohistochemical staining for CD31 and quantification of CD31 positive areas ($n = 6$). (D) The mRNA expression of *VEGF* and *VEGFR* was assessed by qRT-PCR ($n = 6$). *$P < 0.05$

fibroblasts is associated with various factors. Activation of the ERK pathway upregulates the expression of the α1(I) collagen gene [29]. In addition, the ERK and p38 kinase cascades play important roles in wound contraction in rat fibroblasts [30]. Similarly, the PI3K-AKT pathway is involved in the regulation of cell viability in response to mechanical forces in skin fibroblasts [31]. In keloids, fibroproliferation is regulated in part by the MEK-ERK pathway in association with PI3K-AKT signaling activation [32]. These studies suggested the involvement of the AKT/ERK signaling in KF proliferation associated with keloids, providing a rationale for the mechanism underlying the antiproliferative and collagen-inhibiting effect of GA demonstrated in the present study.

In previous work from our group, we showed that growth differentiation factor 9 promotes KF proliferation and migration in part by upregulating MMP-2 and MMP-9 [33]. Fibroblasts are involved in tissue remodeling and wound healing, and the balance between MMPs and TIMPs is critical for ECM remodeling during wound healing [34]. Imbalances between TIMPs and MMPs are implicated in pathologic fibrosing diseases of the skin, including hypertrophic scars and keloids [35]. MMP-2 expression in keloids is higher in collagen bundle regions than in non-collagen bundle regions, and MMP-2 activity and expression are increased in KFs, suggesting that MMPs are involved in the remodeling of collagen bundle regions and invasion of fibroblasts into peripheral regions by promoting the degradation of the ECM [3]. Consistent with these findings, siRNA-mediated inhibition of TIMP-1 in KFs increased the degradation of collagen type I in the culture supernatant in parallel with the inhibition of cell viability and induction of apoptosis [36]. Our results showed that GA inhibited the migration of KFs, downregulated the expression of MMP-1 and MMP-3, while upregulated the expression of TIMP-1. Taken together, these results suggest that targeting MMPs and TIMPs is a potential strategy for the treatment of keloids by inducing the degradation of collagen bundles. Our results support this notion and suggest a potential mechanism underlying the effect of GA mediated by the downregulation of MMPs.

To further examine the effect of GA, we generated *ex vivo* explant cultures of keloid tissues and exposed them to different concentrations of GA. Our results showed that GA inhibited the migration of KFs to the wound area, inhibited collagen deposition, and suppressed angiogenesis. Angiogenesis and the expression and activity of vascular factors are important for the progression of keloids, and endogenous TGF-β1 and VEGF promote angiogenesis in keloids [37,38]. Although keloids are considered inflammatory scars, the pathogenesis of keloids is suggested to involve endothelial dysfunction associated with vascular disorders, and the inhibition of angiogenesis is therefore a potential therapeutic approach [39]. Our results showing that GA inhibits the expression of angiogenic markers are important and support the potential therapeutic application of GA in the treatment of keloids.

In conclusion, we showed that GA inhibited KF proliferation, migration and invasion and induced apoptosis, and the effects of GA were mediated at least in part by the inhibition of the AKT/ERK signaling, the downregulation of MMPs, and the suppression of angiogenesis. These results provide potential mechanisms underlying the effect of GA on KFs and support the potential of GA as a therapeutic approach for the treatment of keloids.

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