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Gallic acid attenuates TGF-β1-stimulated collagen gel contraction via suppression of RhoA/Rho-kinase pathway in hypertrophic scar fibroblasts

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ABSTRACT

Aims: To examine the effect and molecular mechanism of gallic acid (GA) on transforming growth factor-β1 (TGF-β1)-stimulated hypertrophic scar fibroblast (HSF) contraction.
Materials and methods: A fibroblast-populated collagen lattice (FPCL) was developed to examine the effect of GA on TGF-β1-enhanced HSF contraction. The changes in crucial factors related to cell contraction including α-smooth muscle actin (α-SMA), F-actin, and the phosphorylation level of myosin light chain (MLC) were evaluated using western blot and immunostaining. The activation and expression of RhoA/ROCK after the TGF-β1 challenge and GA insult were evaluated using RhoA-G-LISA and RhoA-ELISA kit while the phosphorylation level of MYPT1 and the expression of ROCK1 and ROCK2 were examined by western blot, respectively.
Key findings: GA significantly suppressed TGF-β1-stimulated HSF contraction in a dose- and time-dependent manner. Moreover, the TGF-β1-enhanced α-SMA expression, F-actin formation, and MLC phosphorylation were obviously attenuated by GA. TGF-β1 significantly stimulated RhoA activation but did not alter the expression of RhoA in the HSFs. However, both the activation and expression of RhoA decreased obviously with GA pretreatment followed by TGF-β1 stimulation. Furthermore, GA inhibited ROCK activity but did not affect its expression after TGF-β1 stimulation.
Significance: These results suggest that GA exhibited the potential to prevent HSF contraction after TGF-β1 stimulation by down regulating the RhoA/ROCK signal cascade, followed by the inhibition of the expression of α-SMA, F-actin formation, and phosphorylation of MLC.

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1. Introduction

Hypertrophic scarring is characterized by fibroblast overproliferation during wound healing. In the process of wound healing, fibroblasts play a crucial role including migrating to the wound site, proliferation, synthesis of extracellular matrix, and transforming into α-smooth muscle actin (α-SMA) containing myofibroblasts to facilitate wound contraction and wound closure [1]. Finally, wound healing is completed by myofibroblasts preceding apoptosis.

However, especially with burns and trauma covering large areas, the fibroblasts often over-proliferate and fail to undergo apoptosis, which leads to excessive wound contraction or contractures [2]. Undesirable wound contraction could be detrimental and create cosmetic and functional problems for patients such as pain, cosmetic disfiguration, deformity, and potential loss of function, especially that of the joint mobility. However, the mechanism underlying scar contraction remains unclear. Furthermore, consistent and effective treatment for scars and scar contractions are not available.

Three crucial factors contribute to cell contraction including α-SMA expression, F-actin assembly, and myosin light chain (MLC) phosphorylation [3–6]. RhoA/Rho-kinase (ROCK) signaling was reported to be involved in a variety of actin-based cellular processes including smooth muscle contraction, cell migration, and stress fiber formation. RhoA acts as a molecular switch. It activates downstream effectors to execute

Abbreviations: α-SMA, α-smooth muscle actin; ELISA, enzyme-linked immunosorbent assay; F-actin, filamentous actin; FPCL, fibroblasts populated collagen lattice; GA, gallic acid; G-LISA, GTPase-linked immunosorbent assay; HSFs, hypertrophic scar fibroblasts; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, Myosin phosphatase target subunit 1; ROCK, Rho-kinase; TGF-β1, Transforming growth factor-β 1.

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its biological effects. ROCK is a major downstream mediator of RhoA. In mammals, ROCKs exists as two isoforms, ROCK1 and ROCK2 [7,8]. Accumulating evidence shows that RhoA/ROCK signaling is a critical regulator of cell contraction by increasing the expression of α-SMA [9,10], F-actin [11], and the phosphorylation level of MLC [12,13].

TGF-β1, a well-known pro-fibrotic factor, plays a pivotal role in hypertrophic scar formation. Moreover, TGF-β1 was shown to activate the RhoA/ROCK signaling cascade to increase the expression of these contraction factors and cause cell contraction [14]. Therefore, scar contraction may be inhibited by downregulating the activity of RhoA/ROCK, following the inhibition of the expression of these factors.

Gallic acid (GA) is a naturally occurring plant phenol with diverse biological and pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, anticancer, and antifibrosis effects. A previous study conducted by Phan et al. [15] confirmed that GA inhibits collagen gel contraction in hypertrophic scar fibroblasts (HSFs), but the underlying mechanism remains unclear. In addition, GA was shown to suppress the expression of RhoA and F-actin polymerization in gastric cancer cell metastasis [16,17]. Therefore, we investigated whether GA attenuates HSF contraction by down regulating the expression of RhoA/ROCK, thereby decreasing the expression of α-SMA, F-actin and the phosphorylation of MLC.

2. Materials and methods

2.1. Chemicals

Collagen was purchased from Corning Co. (NY, USA). Gallic acid (GA) and anti-α-SMA antibody (A2547) were purchased from Sigma Co. (St. Louis, MO, USA). Anti-p-MLC (#3675) and anti-MYPT1 (#2634) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-MYPT1 (Thr696) (ABS45) was purchased from Merck Millipore (Darmstadt, Germany). Anti-ROCK1 (sc-5560), anti-ROCK2 (sc-5561) and anti-β-actin antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). RhoA GTPase-linked immunosorbent assay (G-LISA) kit, RhoA enzyme-linked immunosorbent assay (ELISA) kit and Rhodamine labeled phalloidin were purchased from cytoskeleton Inc. (Acoma, CO, USA).

2.2. Fibroblasts isolation and cell culture

Hypertrophic scar specimens were obtained from patients who had not received previous treatment. Informed consent was obtained from the patients and all procedures involving patients were approved by the Research Ethical Committee of Taichung Veterans General Hospital in adherence to the Helsinki Principles. Scar tissue was cut into 1–2 mm pieces. The pieces were then placed in 6-well cell culture plate and added 2 ml Dulbecco's Modified Eagle Medium with 100 U/ml penicillin and 100 mg/ml streptomycin and 10% fetal bovine serum at 37 °C in air containing 5% CO_2. The medium was changed every three days. When fibroblasts reached 90% confluence, cells were subcultured with 0.25% trypsin and 0.04% ethylenediaminetetraacetic acid (EDTA). Only cells from passages fourth to tenth passages were analyzed in this study. Each experiment was repeated three or four times.

2.3. Collagen gel contraction assay

The contraction assay was performed to evaluated the contractility of the hypertrophic scar-derived fibroblasts using hydrated collagen gel lattices on 24-well culture plates as described elsewhere [18,19]. Briefly, fibroblasts were treated with trypsin-EDTA, washed with DMEM, and re-suspended in DMEM, following mixed with collagen solution. The final concentration of collagen was 1.0 mg/ml with a cell density of $2.0 * 10^5$ cells/ml. The mixture (0.5 ml) was added to each well of a 24-well culture plate and allowed to polymerize for 1 h at 37 °C. After gelatinization, another 0.5 ml of serum-free DMEM was poured onto the gel to prevent the surface from dehydrating. After stimulating with or without TGF-β1 (5 ng/ml), in the presence or absence of GA, the digital photos of each gel sample were obtained by screening at 0, 24, 48, 72 and 96 h. Then, the surface area was calculated using the pixel calculation tool of Corel Photo Paint. The relative contraction ratio was evaluated and was presented as a percentage of each treatment area compared with control area. In the ROCK inhibitor treatment, the HSF was treated with Y-27632 for 30 min prior to the TGF-β1 treatment followed by same procedure described above.

2.4. Cell viability in collagen gels

To exclude the effect of cell cytotoxicity on the collagen gel contraction, the viable cell number in the collagen gels was counted. After treatment with TGF-β1 in the presence or absence of GA for 24 h, the medium was replaced with 0.5 ml of serum-free DMEM containing 1 mg collagenase and incubated at 37 °C until the collagen gels were dissolved. The HSFs were sedimented and resuspended in complete medium. The viable cell number was assessed using trypan blue exclusion method.

Western blot analysis

HSFs were treated with TGF-β1 in the presence or absence of GA for indicated time. After reaction, the cells were harvested and frozen to −70 °C immediately. Protein was extracted by lysing frozen cells with cold RIPA (mainly containing 1% Noidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Aprotine, and 100 mM PMSF) and incubated on ice for 20 min. The lysates were then centrifuged 12,000 rpm at 4 °C for another 20 min, and then the supernatants were harvested. After quantity and thermal denatured at 95 °C for 10 min, samples containing equal amounts of protein were resolved by SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked for 1 h at room temperature with 5% milk. The membrane was then incubated with primary antibody at 4 °C, overnight. Followed, the membrane was incubated with secondary antibody at room temperature for 1 h. β-actin was used as an internal standard to ensure equal loading. The relative expression of protein level was compared with the control group.

2.5. Immunofluorescence and phalloidin staining

After treatment with TGF-β1 in the presence or absence of GA for indicated time periods (48 or 96 h), HSFs were fixed in 4% paraformaldehyde for 30 min, following cells were washed with PBS three times for 5 min and were subjected to block and penetrate using PBS with 2% BSA and 0.5% Triton X-100 for 1 h at room temperature. For phalloidin staining, fixed cells were incubated with rhodamine-labeled phalloidin in 1:100 dilution for 1 h. For α-SMA staining, fixed cells were incubated with monoclonal anti-α-SMA (1:200, Sigma) for 2 h at room temperature. After wash with PBS three times, the cells were incubated with FITC-conjugated secondary antibody for 1 h, which was followed by another three washes with PBS for 5 min. Sample was observed using laser-scanning confocal.

2.6. ELISA and G-LISA assay

After treatment with TGF-β1 in the presence or absence of GA for indicated times, HSFs were lysed and stored in liquid nitrogen immediately. Equal amount of protein were used to examine the expression and activation of RhoA protein using enzyme-linked immunosorbent assay (ELISA) and GTPase-linked immunosorbent assay (G-LISA) kit of RhoA (Cytoskeleton, Inc., USA) according to the manufacturer's instructions.

2.7. Statistical analysis

All experiments were performed in triplicate and were repeated at least three separate runs of the experiment. Western blot bands were

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