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Tolfenamic Acid Induces Cell Apoptosis and Inhibits Collagen Accumulation in Keloid Fibroblasts

Dan Yi
Wright State University

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TOLFENAMIC ACID INDUCES CELL APOPTOSIS AND INHIBITS COLLAGEN ACCUMULATION IN KELOID FIBROBLASTS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

DAN YI
B.S., Chongqing Medical University, 2011

2013
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Dan Yi ENTITLED Tolfenamic Acid Induces Cell Apoptosis and Inhibits Collagen Accumulation in Keloid Fibroblasts BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Richard Simman, M.D.
Thesis Director

Norma C. Adragna, Ph.D.,
Interim Chair, Department of Pharmacology and Toxicology

Committee on Final Examination

Richard Simman, M.D.

Yanfang Chen, M.D., Ph.D.

David. R. Cool, Ph.D.

R. William Ayres, Ph.D.
Interim Dean, Graduate School
ABSTRACT

Dan Yi, M.S. Department of Pharmacology and Toxicology, Wright State University, 2013. Tolfenamic Acid Induces Cell Apoptosis and Inhibits Collagen Accumulation in Keloid Fibroblasts.

Keloid scar is a fibroproliferative disorder which results from the accumulation of collagen through the activation of TGF-ß/Smad signaling. Tolfenamic acid (TA) has been found to inhibit collagen synthesis in animal and induce cancer cell apoptosis. In this study, we investigated the effect of DMSO on cell apoptosis, TA on collagen deposition, and TA on collagen type I and smad2 protein expression in human normal fibroblasts (NFs) and human keloid fibroblasts (KFs). In the dose- and exposure time- response assays, we found that 1% DMSO for 48h treatment did not obviously induce NF and KF apoptosis and that 0.55x10^{-3} M TA for 48h treatment remarkably decreased NF and KF proliferation. Under these experimental conditions, we further found that TA selectively induced cell apoptosis, strikingly decreased collagen production, and effectively inhibited collagen type I expression in KFs. Our data suggested that TA induces cell apoptosis and inhibits collagen accumulation in KFs.
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<td>KFs</td>
<td>Human keloid fibroblasts</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>Smad</td>
<td>Drosophila similar to mothers against decapentaplegic</td>
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<td>TA</td>
<td>Tolfenamicacid</td>
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<tr>
<td>NASIDs</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>MTT</td>
<td>3-(4,5)-dimethylthiazol(-z-y1)-3,5-di-phenytetrazoliumromide</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle’s Medium</td>
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<td>FACS</td>
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<td>Double-distilled Water</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>PVDF</td>
<td>Polyvinylidenedifluoride</td>
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I. INTRODUCTION

Keloid scar, keloids, (Figure 1) is a raised scar which forms by expanding beyond the boundaries of the original lesion [1]. The main histological manifestation of a keloid scar (Figure 2) is the overgrowth of atypical fibroblasts with excessive accumulation of extracellular matrix components, especially collagen, fibronectin, elastin, and proteoglycans [1-4]. The causes of this type of scar are still unknown, but it has been pointed out that keloid scar can be developed after any dermal abrasion including burns, piercing, or surgery [1-6]. Accompanied with the development of keloids, are pimples, insect bites, scratching, and/or other skin trauma. The clinical symptoms of keloid scar are claw-like growths over normal skin, itching, pain, ulcerate after infection, deformity and joint movement difficulty [5, 6]. Currently, treatments of KFs contain the combination of excision followed by plastic surgery constructive procedures and 5-Fluorouracil with glucocorticoids injections followed by compression therapy using silicone sheets [1, 2]. Nonetheless, the recurrence remains 45%-100% [3, 4]. Moreover, it is difficult to develop animal models that mimic keloids as keloid scars are unique to humans [5]. So the treatment of keloids continue to be a great challenge for the reconstructive surgeon.
Figure 1. Keloid scar formation after left earlobe piercing. The formation of this type of scar behaves like a benign tumor with growing beyond the boundary of its original wound site.
Figure 2. Histological changes of human keloid scar tissue. Comparing with the histological manifestation of human normal dermal tissue, the fibroblasts are over growing with the excessive accumulation of extracellular connective components including collagen, fibronectin, elastin, proteoglycans. 

Normal Wound Healing

Normal wound healing, cicatrisation, is a complicated process for the skin to repair itself after injury [7]. This procedure is a dynamic and interactive process with 4 phases: hemostasis, inflammation, proliferative and tissue remodeling [8, 9]. After injury occurred, thrombocytes deposited at the lesion to form a fibrin clot to control active bleeding in first minutes [7, 8]. Then bacteria and debris are phagocytosed and removed from circulation to reconstruct the barrier built by epidermis and dermis. Next, vascular endothelial cells formed new blood vessels with a new provisional extracellular matrix (ECM) composed of fibroblasts with excreting collagen and fibronectin [8, 10, 11]. Concurrently, epidermis re-epithelialized and the proliferative cells atop the wound bed to cover the new tissue [7]. Finally, new born tissue remodeled with collagen which realigned along tension lines and later unneeded cells were removed by apoptosis [8].

The Formation of Keloid Scar

Differing from normal wound healing, keloid scar formation begins with abnormal tissue growth in the dermal lesion extending beyond the borders of the original wound [9, 12-15]. In contrast, keloid scar mainly occurred with the abnormal fibroplasias and granulation formation of dermis and subcutaneous tissue in wound healing proliferative phase [16]. The pathological wound healing response of keloid scar composed of a high density of mesenchymal cells called keloid fibroblasts (KFs) [13, 14]. Consequently, the over growth of
KFs results in over-abundance of extracellular and intracellular matrix stroma, which is classified by irregularly directed and thick hyalinized spiral bundles described as keloidal collagen [5, 17]. During the formation of keloid scar, the type of collagen initially produced by fibroblasts was type III collagen. Within the maturation of keloid scar, collagen type I gradually replaces collagen type III and eventually formed extracellular matrix with 99% in the wound bed [1, 5, 12-15, 17]. Finally, functionless tissue regeneration after skin trauma known as keloid scar formed [5, 15, 16, 18].

*Keloid Characteristic Changes in TGF-β/Smad Signaling Pathway*

Though the underlying mechanism of keloid scar formation remains unclear, studies have demonstrated the essential role of transforming growth factor-beta (TGF-β) signaling pathway in the pathogenesis of keloid scar [14, 15, 19]. The intracellular and extracellular signaling pathways of keloid scar formation mediated the relevant profibrotic TGF-β responses [13, 16, 20]. Overproduction of intracellular and extracellular matrix through TGF-β signaling activation could be responsible for the occurrence of keloid scar. As a multifunctional cytokine family, TGF-β, activin, and bone morphogenetic protein, play crucial roles in regulating cellular responses such as proliferation, differentiation, immigration, apoptosis and biosynthesis of connective tissue [16, 20]. Dysregulation of the signaling transduction could cause a number of human diseases, e.g. fibrosis, vascular disorders, cancer, etc [5, 12, 21]. In TGF-β signaling pathway, TGF-β activates TGF-β receptors to promote its downstream effective molecules, including members of the Drosophila Similar
to Mothers Against Decapentaplegic (Smad) family (Figure 3) [9, 18]. The smad family which is classified as receptor-regulated Smads (R-Smads: Smad1, 2, 4, 5 and 8), common mediator Smad (Co-Smad: Smad4), and inhibitory Smads (I-Smads: Smad6 and 7) is one of the dominant intracellular signaling mediators activated by TGF-β receptors [18]. In TGF-β signaling pathway, smad2 and smad3 are two critical conductive factors affecting nuclear gene expression [5, 16, 21, 22]. The involvement of TGF-β/Smad signaling on keloid pathogenesis has been illustrated in some studies [16, 21, 22]. It has been pointed out that smad family is involved in pathological fibrosis through phosphorylating Smad2/3 hetero-oligomeric complex which is consequently translocated from the cytoplasm to the nucleus to affect the synthesis and expression of collagen type I [12, 19]. In addition, smad pathway has also been shown to influence the expression of collagen type I in human glomerular mesangial cells and human mesangial cells [12, 19, 23]. Thus, suppressing smad2 expression could contribute to abnormal TGF-β responses of fibroproliferation in keloid scar [5, 17, 19, 21, 23]. Therefore, the TGF-β/Smad signaling pathway is a potential target for treating keloid fibroblasts.
Figure 3. TGF-β/Smad signaling pathway plays an essential role in regulating collagen production in keloid fibroblasts. In keloid scar, TGF-β signaling are upregulated. Representatives of smad family such as smad2, smad3, and smad4 are the transduction downstream of TGF-β signaling pathway. With the upregulation of TGF-β, the complexes of smad2/3/4 will bind to the nucleus which will cause an increase in gene expression. Meanwhile, the balance between collagen synthesis and degradation is interrupted. Eventually, expressed collagen increases and forms keloid scar with extracellular matrix. Modified from Tuan and Nicher, 1998.
Tolfenamic Acid (TA) is a non-steroidal anti-inflammatory drug (NSAIDs) that is traditionally used for rheumatic disease [24,25]. NSAIDs are non-prescription analgesics and antipyretics which are available over the counter in most countries [25, 26]. The representative members of NSAIDs are aspirin, ibuprofen, and naproxen, all of which are used as a non-addictive alternative to narcotics [26]. The dominate medical use of this group of drug are rheumatoid arthritis, osteoarthritis, Inflammatory arthropathies, acute gout, dysmenorrhoea, metastatic bone pain, headache and migraine, postoperative pain, mild-to-moderate pain due to inflammation and tissue injury, muscle stiffness and pain due to Parkinson's disease, pyrexia, renal colic, etc [27-30]. The main adverse effects of NSAIDs are gastrointestinal and renal effects such as ulcer perforation, upper gastrointestinal bleeding, nausea, vomiting, dyspepsia, diarrhea, salt and fluid retention, hypertension, interstitial nephritis, nephritic syndrome, acute renal failure, acute tubular necrosis, etc [30, 31]. Alike many medicine, there are drug interactions between NSAIDs and others: inhibiting the elimination of lithium and methotrexate when using with diuretics, causing severe hypoocoagulability when combining with warfarin, could aggravate hypertension when adding with antihypertensives, could interfere and reduce efficiency of antidepressants, etc [28, 30-32]. Majority of NSAIDs are functional as non-selectively inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes [33]. COX-1 is a constitutively expressed "house-keeping" enzyme which regulates many cellular physiological responses [26, 34]. COX-2 is an enzyme expressed to
down-regulate inflammation symptoms [35, 36]. With the deeper studies reported on NSAIDs, it has been divided into different groups: salicylates, propionic acid derivatives, acetic acid derivatives, enolic acid derivatives; fenamic acid derivatives, and others. TA belongs to the family of fenamic acid derivatives. TA, 2-([3-Chloro-2-methylphenyl] amino) benzoic acid, with a low solubility in water, it is written out as C_{14}H_{12}ClNO_{2} (Figure 4.) and it has a molecular weight of 261.7 g/mol. The exact medical application, adverse effects, and mechanism of TA are not clear, however, previous studies have shown the special application of TA. Studies illustrated that TA was associated with inhibiting collagen metabolism in connective tissue in rats and had the capacity of inducing cancer cell apoptosis [24, 25, 37-42]. There is a low inhibition of sodium tolfenamate on the metabolism of collagen with 0.15 mol/L NaCl in rats [24]. Additionally, in vascular endothelial growth factor receptor 1-dependent pancreatic cancer cell, cell migration determined by collagen–coated plates was inhibited by TA [42, 43]. TA could reduce cell survival, growth and angiogenesis in tumor and cancer cells, including human xenograft tumor, human pancreatic cancer, human neuroblastoma and mouse prostate cancer by regulating the activity of specific protein 1; human head and neck cancer by regulating non-steroidal anti-inflammatory drug activated gene-1; human colorectal cancer through affecting E-twenty six metazoans family protein epithelial specific transcription factor-1/early growth response-1 performance, and human oral cancer by affecting p38 mitogen-activated protein kinases signaling pathway expression [25, 37-41].
Figure 4. Structure of tolfenamic acid.

Adapted from Beetstra, 2009.
II. HYPOTHESIS AND AIMS

Due to the complicated therapeutic methods with a high reoccurrence of keloid scar in clinical treatment, new advanced treatment is clamant [1-4]. Even though many questions still remain in regards to TA, TA has been suggested as a kind of NSAIDs with high therapeutic index which enjoys a promising long-term treatment in treating osteoarthritis, rheumatoid arthritis, migraine, and cancer [24, 25, 37-42]. Moreover, as TA is insoluble in water, dimethyl sulfoxide (DMSO) was selected as vehicle to dissolve TA for most studies [25, 37-41]. However, the effect of DMSO was not particularly illustrated based on those reports. Thus far, it is very necessary to explore the potential effect of DMSO on treating KFS.

This study is to determine the capacity of TA on treating KFs including cell proliferation, cell apoptosis, collagen deposition, and collagen type I and smad2 protein expression comparing with NFs. Our ultimate goal is to gain the information from this project for clinical application to determine if TA is a potential therapy for keloid scar. The expecting benefits for the patients may contain faster healing time of chronic wound bed with less recurrence thereby reducing medical costs of repetitive remedies and surgeries and finally elevating the life quality of patients.
**Overall Hypothesis**

We hypothesizes that TA will decrease cell proliferation, induce cell apoptosis, and inhibit collagen accumulation of collagen in KFs by down-regulating the TGF-β/Smad signaling.

**Specific Aims**

1. To characterize the effect of DMSO on NF and KF apoptosis.
2. To characterize the effect of TA on NF and KF proliferation.
3. To test the effect of TA on NF and KF apoptosis.
4. To test the effect of TA on NF and KF collagen accumulation.
5. To determine the effect of TA on the expression of collagen type I in NFs and KFs.
6. To explore the influence of TA on the expression of smad2 protein in NFs and KFs
III. EXPERIMENTAL DESIGNS

To test my hypothesis, I have designed my experimental procedure in a flow chart (Figure 5.). Before testing all the steps, I abstracted NFs and KFs from tissue samples and incubated them separately. To test specific aim 1, I first treated NFs and KFs with different concentration of DMSO for different exposure time, respectively. Then I analyzed cell apoptosis caused by each concentration and exposure time of DMSO through Annexin V/Propidium Iodide (PI) labeling. To test specific aim 2, I treated NFs and KFs with different doses of TA for different exposure time, respectively. Next, I analyzed cell proliferation after each concentration and exposure time treatment by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) staining. Based on specific aim 1 and 2, it was expected that there is a desirable concentration and exposure time of DMSO that is less effective on NFs and KFs, and there is a desirable concentration and exposure time of TA that is more effective on KFs. By using this particular concentration of DMSO to dissolve TA and use the designated treatment time of TA on KFs, I continued to test specific aim 3 in cell apoptosis by Annexin V/PI labeling, specific aim 4 in collagen production by Sirius Red Staining, specific aim 5 in the expression of collagen type I and specific aim 6 in the expression of smad2 protein by Western Blot.
Figure 5. Experimental design based on my hypothesis and specific aims.
IV. MATERIALS AND METHODS

Cell Culture and Treatment

The protocol for obtaining skin tissues during plastic surgeries was approved by the ethical institutional review board of Wight State University. Patients signed the consent forms. The keloid scar tissue was taken from a 24 year old Africa-American with clinical and pathologic evidences of keloid scar confirmed as previously described [14, 16]. The normal adult skin samples were obtained from a 29 year old Africa-American during plastic surgery [9, 12]. Skin specimens were first washed with phosphate-buffered saline (PBS) (Gibco, Life Technologies™, USA) three times to exclude erythrocytes, then cut into 2.5mm x 2.5mm pieces and incubated with 2 ml digestion medium containing high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies™, USA),5 mg/mL collagenase/dispase II (RocheDignotics, USA) and 0.25% trypsin (Invitrogen, Life Technologies™, USA) in 60x15mm tissue culture dish for 8h at the condition of 5% CO₂, at 37°C [9, 13, 19]. Then the tissue suspension was passed through a 100 mm filter (Biologix Research Company, USA) to remove any remaining tissue residuals. The suspension was next centrifuged at 300 G for 8 min to obtain cell pellet. This pellet was transferred to a T25 cell culture flask cultured with 3 ml total medium containing high glucose DMEM, 10%FBS (Gibco, Life Technologies™,USA),1% Pen/Strep/Glutamine (Invitrogen, Life Technologies™, USA) in the
condition of 5% CO₂, at 37°C. Cells took approximately 3 days to totally attach to the bottom and reach to confluence in about 10 days. After cells were attached the bottom of the flask, total medium was replaced with fresh total medium. During cell growth, every 2 or 3 days medium was changed after PBS washing to exclude the flatting dead cells. Once reaching confluence (named as Passage 1) cells were detached into a new passage by 0.05% trypsin (Invitrogen, LifeTechnologies™, USA). Passage 2 to passage 6 cell cultures with probably 80%-95% cell confluence were presented in this study [14]. For DMSO effect testing, 6 µl DMSO dissolved in 5994 µl total medium for 0.1% DMSO, 60 µl DMSO dissolved in 5940 µl total medium for 1% DMSO, 600 µl DMSO dissolved in 5400µl total medium for 10% DMSO, and 100% DMSO were pre-prepared. For each assay of TA treatment, TA (Cayman Chemical Company, USA) was measured for 0.2617g and dissolved in 1ml 100% DMSO (Thermo Fisher Scientific, USA) as 1M TA. 900µl 1M TA diluted in 8100µl 100% DMSO as tenfold dilution to 10⁻¹M TA. 150µl 10⁻¹M TA diluted in 15ml total medium to 10⁻³M TA. In cell proliferation analysis, 10⁻³M TA as original concentration was diluted tenfold by total medium, diluted into 10⁻⁴M, 10⁻⁵M, 10⁻⁶M for TA in this order. In cell apoptosis assay, collagen quantification, and western blot of TA treatment group, 3.75ml 10⁻³M TA as the original concentration was diluted by 11.25ml total medium to0.55x10⁻³M TA. In the vehicle treatment group (DMSO group), the same DMSO concentration (0.55%) as in the TA treatment group were used to test vehicle effect.

**Cell Proliferation Analysis**
NFs and KFs (0.1x10^5) were separately seeded into 96-well cell culture plates with 200 µl total medium. After 95% confluence, cells were divided into different groups for pre-treatment with 200 µl total medium with TA (10^{-3}M, 10^{-4}M, 10^{-5}M, or 10^{-6}M) for different periods (24h, 48h, or 72h). The MTT kit (Invitrogen, Life Technologies™, USA) was used for the proliferation assay. Component A in the kit was dissolved by 1 ml PBS to make the MTT solution and then stored at 4°C. Component B in the same kit was dissolved by 10ml 0.01M HCl promptly. After each exposure time, old medium was replaced by 100 ml fresh medium. Next, 10µl of MTT solution was added into each well and incubated for 4h in the condition of 5% CO_2, at 37°C. Then, formazan crystals produced by active cells formed in mitochondria through binding succinate dehydrogenase which were dissolved in 100µl of component B solution for solubilization for 4h in the condition of 5% CO_2, at 37°C. Lastly the absorbance was detected at 535 nm in a Packard Fusion spectrophotometer [19].

Relatives of Cell proliferation (%) = [OD 535(TA treatment group)/ OD 535(Non-TA treatment group)] x100.

**Cell Apoptosis Assay**

NFs and KFs (1.5x10^5) were separately seeded into 12-well tissue culture plates with 2 ml of total medium. For DMSO effect testing, cells with 80% confluence were divided into five different groups for pre-treatment with 2 ml 0%DMSO (total medium), 0.1% DMSO, 1% DMSO, 10% DMSO, or 100% DMSO for 24h, 48h, or 72h, respectively. For TA treatment testing, cells with 85% confluence were divided into different groups for pre-treatment with 2 ml...
total medium (Control, Vehicle, and TA) for 48h. Preparing 1X annexin-binding buffer (Invitrogen, Life Technologies™, USA) and 1X PI (Invitrogen, Life Technologies™, USA) working solution through diluting 200 µl 5X annexin-binding buffer with 800 µl DDH₂O (dilution at 1:5) and diluting 5 µl 10X PI working solution with 50 µl annexin-binding buffer (dilution at 1:10) then all solutions were stored at 4°C. After each exposure time for different testing group, cells in each well were washed with 2 ml PBS twice and detached by 300 µl 0.05% trypsin for 1 min followed by adding 700 µl total medium to stop the detachment. Then cells in each well were centrifuged at 400G for 9 min to produce a cell pellet. Annexin-binding buffer 100 µl 1X was used to suspend the pellet. Suspended cells were stained with 5 µl AnnexinV solution (Invitrogen, Life Technologies™, USA) and 1 µl PI working solution for 15-20 min labeling in the dark. After labeling time, cell apoptosis were detected by fluorescence-activated cell sorting (FACS) Calibur Flow Cytometer as soon as possible (Accuri C6, Inc., USA), with 488 nm excitation and 530 nm emission [38, 39].

**Collagen Quantification**

NFs and KFs (1.0x10⁵) were separately seeded into 24-well cell culture plates with 1 ml total medium. After 80-95% confluence, cells were divided into different groups for pre-treatment with 1 ml total medium (Control, Vehicle, and TA) for 48h. To analyze collagen production, Sirius Red/Fast Green Staining kit (Chondrex, USA) was presented in this assay. After the exposure time, culture medium was removed and the cells were washed with PBS. Next, cooled 95%
0.5 ml ethanol was added (Decon, Labs, Inc. USA) into each well and incubated for 15 minutes at room temperature to fix the cells. Dye solution0.2ml from the kit was added into ethanol layers which completely immersed the fixed layer for staining at room temperature for 1-1.5h. The dye solution was removed and the cell layers were rinsed with DDH₂O for six times and observed under a microscope. After microscope observation, the DDH₂O were replaced with 1 ml of dye extraction solution from the kit and gently mixed until the color was eluted from the cell culture layer. The absorbance at 535 nm and 600 nm were read with a Packard Fusion spectrophotometer. Equation: Collagen (μg/well) = [OD 535-(OD 600 x 0.291)]/0.037 (Chondrex, USA). Relatives of Collagen quantification (%) = [collagen amount (each group in NFs or KFs)/ collagen amount (NFs control group)] x 100.

**Western Blot Analysis**

NFs and KFs (2.5x10⁵) were separately seeded into T25 cell culture flasks with 3ml total medium. After 85% confluence cells were divided into different groups for pre-treatment with 3 ml total medium (Control, Vehicle, and TA) for 48h. 1tablet of protease inhibitors was added (Roche Diagnostics GmbH, USA) into 10ml Lysis buffer (Thermo Fisher Scientific, USA) and stored at 4°C. 8%SDS-PAGE separating gel and 10% SDS-PAGE gel were used for western blot. SDS-PAGE gel was first ran at 80 V for 30 min (Bio-Rad, PowerPac™ HC, USA) as blank running to eliminate the interference caused by gel. Protein concentrations were measured by BCA assay. Sixty micrograms of each sample with different treatment was mixed with 10 μL of 5X protein sample
loading buffer. After loading the samples, proteins were presented at 80 V until all the markers appeared. The PVDF membrane was activated initially with 100% methanol for 15 min, and washed with DDH$_2$O for 1 min, then stored in transfer buffer for 10 min. The transfer process from gel to membrane was conducted at constant 50mA overnight. The trans-blotted PVDF membrane was blocked with blocking buffer (3% BSA; 1X TBS; and 0.05% Tween-20) for 1 h. It was then incubated with primary antibodies rabbit monoclonal anti-human collagen type I (dilution 1:2000, Thermo Fisher Scientific, USA), and rabbit monoclonal anti-human Smad2/3 (dilution 1:800, Thermo Fisher Scientific, USA) separately for over two nights at 4°C, then washed with TBS-T for three times for 5 min each. Anti-rabbit HRP-conjugated secondary antibody (dilution at 1:20000, Sigma, USA) was added after washing and incubated for another 1 h at room temperature. Next, the membrane was washed in TBST for three times for 5 minutes each. The membrane was incubated with chemiluminescent HRP substrate (Cell Signaling Technology, USA) for 4 min in the dark at room temperature before being visualized and then quantified using a chemiluminescent detection system (ECL; Bio-Rad, Chemidoc™ MP, USA). Protein band intensity in each lane was scored in volume intensity and was normalized with beta-actin (dilution at 1:4000, Sigma, USA) to standardize protein loading[19].

**Statistical Analysis**

All experiments were performed independently at either four or six times with keloid scar culture as well as normal skin dermal culture. Data were analyzed
by STATISTICA version 6.0 software package (StatSoft, Inc. USA) and expressed as mean ± SEM which was evaluated by applying one-way ANOVA between two groups in this study. \( P<0.05 \) was considered statistically significant for all tests.
V. RESULTS

The Dose and Exposure Time Responses of DMSO on Cell Apoptosis in NFs and KFs

To determine whether DMSO could induce cell apoptosis on NFs and KFs, we performed Annexin-V/PI labeling of fluorescence-activated cell sorting (FACS) after applying 0%, 0.1%, 1%, 10%, or 100% DMSO for 24, 48, or 72 hours, respectively. Table 1 and Table 2 showed the average events counted in DMSO treatment on NF and KF apoptosis. For each analysis, total 10000 events were counted. The living cells were defined as Annexin V (-) and PI (-); early apoptotic cells were defined as Annexin V (+) and PI (-); late apoptotic cells were defined as Annexin V (-) and PI (+); dead cells were defined as Annexin V (+) and PI (+). From Figure 6, with the addition of concentration of DMSO, the proportion of early apoptotic and late apoptotic cells were increased. Compared with 0% DMSO (control), only 100% DMSO induced both early and late apoptosis significantly in NFs. After 48h treatment (Figure 7), 10% and 100% DMSO strikingly induced a significant early and late apoptosis in NFs. For 72h DMSO treatment group (Figure 8), cell apoptosis in 1%, 10% or 100% DMSO treatment were increased, especially the percentage of early apoptosis. With the addition of exposure time increased (Figure 9), 1% DMSO after 72h treatment induced an obvious cell apoptosis compared to 48h and 24h in NFs. From Figure 10, with the increasing concentration of DMSO,
The proportion of early apoptotic and late apoptotic cells were increased in KFs. Compared with 0% DMSO (control), 10% and 100% DMSO induced both early and late apoptosis significantly. After 48h treatment (Figure 11), only 100% DMSO induced a remarkable early and late apoptosis in KFs. For 72h DMSO treatment (Figure 12), 1%, 10% and 100% DMSO increased a striking apoptosis percentage, especially the percentage of early apoptosis. With the exposure time increased (Figure 13). 1% DMSO group for 72h treatment induced an obvious cell apoptosis compared with 24h and 48h in KFs. The two figures demonstrated that cell apoptosis of NFs and KFs were induced by DMSO in a dose- and exposure time- response way. Lower concentration of DMSO (0.1% and 1%) with less exposure time (24h and 48h) did not significantly induce cell apoptosis in NFs and KFs. The presented data were based on four independent experiments, comparing with non-DMSO treatment group in NFs and KFs separately.
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Table 1. Average events counted of DMSO on cell apoptosis in NFs. For each analysis, total 10000 events were counted. The living cells were defined as Annexin V (-) and PI (-); early apoptotic cells were defined as Annexin V (+) and PI (-); late apoptotic cells were defined as Annexin V (-) and PI (+); dead cells were defined as Annexin V (+) and PI (+).
Figure 6. The dose responses of DMSO for 24h on cell apoptosis in NFs. 100% DMSO with 24h exposure time induced a significant early and late apoptosis in NFs. 0: 0% DMSO, total medium; 0.1, 0.1% DMSO; 1: 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. *, P<0.05, 100 vs. 0.1; n=4.
Figure 7. The dose responses of DMSO for 48h on cell apoptosis in NFs. 10% and 100% DMSO with 48h treatment induced both early and late apoptosis in NFs, respectively. 0: 0% DMSO, total medium; 0.1: 0.1% DMSO; 1: 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. +, P<0.01, 10 vs. 0, 0.1, or 1; &, P<0.01, 100 vs. 0, 0.1, 1, or 10; n=4.
Figure 8. The dose responses of DMSO for 72h on cell apoptosis in NFs. 1%, 10%, and 100% DMSO induced a significant cell apoptosis especially the percentage of early apoptosis in NFs. 0: 0% DMSO, total medium; 0.1: 0.1% DMSO; 1: 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. $, P<0.01, 1 vs. c or 0.1; *, P<0.01, 10 vs. c, 0.1, or 1; $, P<0.01, 100 vs. c, 0.1, or 1; n=4.
Figure 9. The exposure time responses of 1% DMSO on cell apoptosis in NFs.

1% DMSO with 48h and 72h exposure time induced cell apoptosis in NFs. 1% DMSO with 72h exposure time induced an obvious cell apoptosis especially early apoptosis in NFs. ※※, P<0.01, 72h vs. 24h or 48h; n=4.
Table 2. Average events counted of DMSO on cell apoptosis in KFs. For each analysis, total 10000 events were counted. The living cells were defined as Annexin V (-) and PI (-); early apoptotic cells were defined as Annexin V (+) and PI (-); late apoptotic cells were defined as Annexin V (-) and PI (+); dead cells were defined as Annexin V (+) and PI (+).
Figure 10. The dose responses of DMSO for 24h on cell apoptosis in KFs. 10% and 100% DMSO with 24h exposure time induced a significant early and late apoptosis in KFs. 0: 0% DMSO, total medium; 0.1: 0.1% DMSO; 1: 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. *, P<0.01, 10 vs. c, 0.1, or 1; #, P<0.01, 100 vs. c, 0.1, 1 or 10; n=4.
Figure 11. The dose responses of DMSO for 48h on cell apoptosis in KFs. 100% DMSO with 48h exposure time induced a significant early and late apoptosis in KFs. 0: 0% DMSO, total medium; 0.1: 0.1% DMSO; 1: 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. +, P<0.05, 100 vs. c, 0.1, 1, or 10; n=4.
Figure 12. The dose responses of DMSO for 72h on cell apoptosis in KFs. 1% and 10% DMSO with 72h exposure time induced a significant early and late apoptosis in KFs. 100% DMSO with 72h exposure time induced a significant early apoptosis in KFs. 0% DMSO: total medium; 0.1% DMSO; 1% DMSO; 10: 10% DMSO; 100: 100% DMSO. &, P<0.01, 1 vs. c or 0.1; $, P<0.01, 10 vs. c, 0.1, or 1; *, P<0.01, 100 vs. c, 0.1, 1, or 10; n=4.
Figure 13. The time responses of 1% DMSO on cell apoptosis in KFs. 1% DMSO after 48h and 72h will induce cell apoptosis in KFs. 1% DMSO with 72h exposure time induced an obvious early and late apoptosis in KFs. ※※, P<0.01, 72h vs. 24h or 48h; n=4.
The Dose and Exposure Time Responses of TA on Cell Proliferation in NFs and KFs

The effect of TA on NFs and KFs proliferation was evaluated using MTT analysis. NFs and KFs were cultured in the presence of TA (10⁻³M, 10⁻⁴M, 10⁻⁵M, 10⁻⁶M) dissolved in 1% DMSO for 24, 48, or 72 hours, respectively. From Figure 14 and 15, at the highest concentration of TA, cell proliferation rates appeared to decrease. As the exposure time increased, NF proliferation sharply decreased at 10⁻³M TA. More specifically, as shown in Figure 14., the proliferation rate was significantly reduced in NFs at 10⁻³M TA for 48h or 72h group comparing at 24h. In KFs, there is no statistical difference on the cell proliferation rate between the control group and TA-treated groups in 24h testing group (Figure 15.). While after 48h or 72h treatment of TA on KFs with the addition of TA, the proliferation rate decreased at 10⁻³M TA. However, this concentration decreased cell proliferation in both NFs and KFs as well. To further test the effects of TA on NFs and KFs, we selected 0.55x10⁻³M TA with 48h exposure time as our testing dosage and exposure time. The two figures demonstrated that cell proliferation of NFs and KFs were decreased by more than 10⁻⁴M of TA with 48 or 72 exposure time. The presented data were based on six independent experiments, comparing with non-TA treatment group in NFs and KFs separately.
Figure 14. The dose and exposure time responses of TA on cell proliferation in NFs. In 48h exposure time group and 72h exposure time group, $10^{-3}$M TA significantly decreased cell proliferation in NFs. In $10^{-3}$M TA treatment group, treatment with 48h and 72h exposure time decreased cell proliferation significantly. 0, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, 0.55x$10^{-3}$: M TA was dissolved in 1% DMSO. *, P<0.01, $10^{-3}$M vs. 0M; +, P<0.01, 48h vs. 24h; #, P<0.01, $10^{-3}$M vs. 0M or $10^{-4}$M; &, P<0.01, 72h vs. 24h or 48h; n=6.
Figure 15. The dose and exposure time responses of TA on cell proliferation in KFs. In 48h exposure time group and 72h exposure time group, $10^{-3}$M TA significantly decreased cell proliferation in KFs. In $10^{-3}$M TA treatment group, treatment with 48h and 72h exposure time decreased cell proliferation significantly. 0, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, 0.55x$10^{-3}$ M TA was dissolved in 1% DMSO. **, $P<0.01$, $10^{-3}$M vs. 0M; ++, $P<0.05$, 48h vs. 24h; ##, $P<0.05$, $10^{-3}$M vs. 0M; &&, $P<0.01$, 72h vs. 24h or 48h; n=6.
The Effects of TA on cell apoptosis in NFs and KFs

To determine whether TA induced cell apoptosis on NFs and KFs, we performed Annexin-V/PI labeling of FACS after applying $0.55 \times 10^{-3}$MTA dissolved in 1% DMSO for 48h. Table 3 showed the average events counted in TA treatment on NF and KF apoptosis. For each analysis, total 10000 events were counted. The living cells were defined as Annexin V (-) and PI (-); early apoptotic cells were defined as Annexin V (+) and PI (-); late apoptotic cells were defined as Annexin V (-) and PI (+); dead cells were defined as Annexin V (+) and PI (+). According to Figure 16, Annexin V positive and PI positive cells were not remarkably increased by TA in NFs while they were significantly raised in KFs. Though vehicle effect was exhibited in the NFs, it can be pointed out that $0.55 \times 10^{-3}$M TA with 48h exposure time induced selectively significant apoptosis on KFs compared with non-TA treatment groups in KFs and in NFs. Moreover, from Figure 16, we also found that 0.55% DMSO stimulates cell apoptosis in NFs and KFs, especially in NFs. The presented data are based on six independent experiments and compared with non-TA treatment group in NFs.
For each analysis, total 10000 events were counted. The living cells were defined as Annexin V (-) and PI (-); early apoptotic cells were defined as Annexin V (+) and PI (-); late apoptotic cells were defined as Annexin V (-) and PI (+); dead cells were defined as Annexin V (+) and PI (+).

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Table 3. Average events counted of DMSO on cell apoptosis in NFs and KFs.
Figure 16. The effect of TA on cell apoptosis in NFs and KFs. DMSO induced early and late apoptosis in NFs and slightly induced early apoptosis in KFs. TA strikingly induced late apoptosis in KFs. C: control, total medium; V: vehicle,
0.55% DMSO; TA, 0.55x10^{-3}M of TA was dissolved in 0.55%DMSO. *, P<0.01, V vs. C or TA; ##, P<0.05, TA vs. C; &&, P<0.01, TA vs. TA; n=6.

**The Effects of TA on Collagen Production in NFs and KFs**

Quantitative analysis of Sirius Red fluorescent staining at intracellular and extracellular reflects the degree of all types of collagen produced by fibroblasts. Therefore, the intensity of red cell with fluorescence presentation indicated the amount of collagen produced by cells. By using 0.55x10^{-3}M TA on NFs and KFs for 48h, we found that collagen production was significantly reduced in KFs (Figure 17.). According to Figure 17., 0.55% DMSO stimulated collagen production in NFs and KFs, especially in NFs. In other words, TA did not inhibit collagen production significantly in NFs while it obviously decreased the collagen deposition in KFs. Additionally, there was a remarkable observation about morphological changes in KFs. From Figure 18., the staining signals of both intracellular and extracellular were decreased. Stained collagens secreted to extracellular were reduced and meanwhile cytoplasm was concentrated. These changes eventually decreased cell density in KFs. Based on the biases of these data, we demonstrated that 0.55x10^{-3}M TA for 48h reduced collagen production in KFs. The presented data are based on four independent experiments and compared with non-TA treatment group in NFs.
Figure 17. The effect of TA on collagen production in NF and KF after sirius red staining. DMSO significantly increased collagen production in NFs. TA strikingly reduced collagen production in KFs compared with NFs. C: control,
total medium; V: vehicle, 0.55% DMSO; TA, 0.55x10^{-3}M of TA was dissolved in 0.55% DMSO. *, P<0.01, V vs. C; #, P<0.05, TA vs. V; ##, P<0.05, TA vs. C; &&, P<0.01, TA vs. V; n=4.

Figure 18. Morphological changes of TA on collagen production in KFs. Staining signals of intracellular and extracellular were reduced. Stained collagen which secreted to extracellular was decreased and meanwhile cytoplasm was concentrated. Eventually, cell density was decreased. Observed under 20X microscope after sirius red staining.
The Effects of TA on the Expression of Collagen Type I and Smad2 Proteins in NFs and KFs

The effects of TA on protein expression of collagen type I (Collagen I) and smad2 protein in NFs and KFs were evaluated by Western blot. The protein level of Collagen I and smad2 was measured 48 hours later after each treatment. From the view of Figure 19, there was a remarkably increasing expression in KFs compared with NFs. TA significantly suppressed the expression of collagen type I in KFs compared with the other groups; while the inhibition of collagen type I expressed in NFs after TA treatment was ambiguous. This result demonstrated that 0.55×10⁻³ M TA for 48h inhibits collagen I expression significantly in KFs while not effectively in NFs. Besides, 0.55% DMSO slightly decreased the expression of collagen I in NFs and increased collagen I expression in KFs. In Figure 20, TA inhibited Smad2 expression in both NFs and KFs with more significance in KFs. Meanwhile, 0.55% DMSO also reduced the expression of Smad2 protein in NFs group showed in Figure 20. This data suggested that 0.55×10⁻³ M TA for 48h could inhibit smad2 expression in both NFs and KFs. The presented data are based on four independent experiments and compared with non-TA treatment group in NFs.
Figure 19. The effect of TA on the expression of collagen type I in NFs and KFs.

DMSO slightly increased the expression of collagen type I in KFs. TA significantly inhibited the expression of collagen type I in KFs. C: control, total medium; V: vehicle, 0.55% DMSO; TA, 0.55x10^{-3} M of TA was dissolved in 0.55% DMSO. +, P<0.05, C vs. C; &, P<0.05, V vs. NFs C; #, P<0.05, TA vs. V; n=4.
Figure 20. The effect of TA on the expression of smad2 protein in NFs and KFs.

DMSO decreased the expression of smad2 protein in NFs. TA significantly inhibited the expression of smad2 protein in KFs. C: control, total medium; V: vehicle, 0.55% DMSO dissolved in total medium; TA, 0.55x10^{-3}M of TA was dissolved in 0.55% DMSO. *, P<0.006, TA vs. C; $, P<0.04, TA vs. TA; n=4.
VI. DISCUSSION

Keloid scar, a type of scars, behaves like tumors with uncontrolled expanding at wound site and invasion to the neighboring normal skin tissue [16, 20]. It has been suggested that keloid scar is a fibroproliferation disease with the accumulation of collagen deposition due to the upregulation of TGF-β/Smad signaling in the wound healing process [5, 14, 16]. TA is a NASIDs which belongs to the family of fenamate and has been used recently for cancer treatments [24, 25, 37-42, 44]. We designed an experiment to determine if the administration of TA is able to decrease collagen production in KFs. We also want to explore the possible mechanism of action of TA in regulating TGF-β/Smad signaling transduction when treating on KFs. Our study demonstrated that TA has the potential to normalize some of the characteristic features of KFs such as cell apoptosis and collagen production especially collagen type I expression.

Various Concentrations and Exposure Time of DMSO Induced Different Apoptotic Results in Human Normal Fibroblasts and Keloid Fibroblast

As TA is insoluble in total medium, a vehicle to dissolve it is certainly necessary. Most studies have illustrated DMSO as a universal solvent [25, 37-41]. However, there is not sufficient data to exclude the effects caused by DMSO. Therefore, the influence of DMSO as a vehicle was conducted in our
study as preliminary experiment. We tested the effect of various concentrations (0%, 0.1%, 1%, 10% and 100%) and exposure times (24h, 48h, and 72h) of DMSO on NF and KF apoptosis. We found that 1% DMSO with 48h exposure time did not significantly induce cell apoptosis. We also found that the least concentration to dissolve experimental dosage of TA ($10^{-3}$M, $10^{-4}$M, $10^{-5}$M, $10^{-6}$M) is 1% DMSO. Thus far, 1% is the experimental concentration of DMSO for continuous studies on KFs. Besides, in our studies, we also set a vehicle group to exclude the influence caused by DMSO.

**Tolfenamic Acid Decreased Cell Proliferation in Human Normal Fibroblasts and Keloid Fibroblasts**

We tested the effects of TA on NF and KF proliferation by applying five concentrations ($10^{-3}$M, $10^{-4}$M, $10^{-5}$M, $10^{-6}$M) of TA for 3 different exposure times (24h, 48h, 72h) separately. We found that NF and KF proliferations were decreased with the $10^{-3}$M TA after 48h exposure. This indicated that cell proliferation was not significantly affected by TA. In addition, under this circumstance, cell proliferation in both NFs and KFs were strikingly reduced. One of our expectations is to find a desirable concentration of TA that could treat KFs effectively and less toxic to NFs. In cell proliferation assay, our data pointed out the concentration which caused half reduction in NF proliferation between $10^{-4}$ and $10^{-3}$M of TA with 48h exposure time as our experimental condition. Though this concentration of TA with 48h exposure time did not show more significant reduction in KFs, we expected there will be more significances presenting in other analysis.
**Tolfenamic Acid Selectively Induced Cell Apoptosis in Human Keloid Fibroblasts**

To further confirm the effects of this particular does and exposure time of TA is the ideal experimental condition, we used $0.55 \times 10^{-3}$M TA with 48h treatment on NFs and KFs to test cell apoptosis. In this concentration of TA, we calculated that there is a 0.55% DMSO as a vehicle, so besides total medium as control group, we set a vehicle group with 0.55% DMSO to test the effects. We found that though 0.55% DMSO induced a proportional apoptosis in NFs and KFs, cell apoptosis in KFs after TA treatment induced a selectively significant apoptosis compared with NFs. This indicated that cell apoptosis was significantly influenced by TA. Thus far, we first suggested that $0.55 \times 10^{-3}$M TA with 48h treatment was the ideal experimental condition for continuous studies on KFs and second TA was able to selectively induce cell apoptosis in KFs.

**Tolfenamic Acid Strikingly Reduced Collagen Production in Human Keloid Fibroblasts**

According to the histological changes in KFs [1-4], reducing collagen production is a targeting way to treating keloid scar. In our study, we tested the effects of TA on decreasing the accumulation of collagen and furthermore tried to explain the relationship between cell apoptosis and collagen deposition. Based on our data, high inhibition rate of collagen production was detected in KFs after $0.55 \times 10^{-3}$M TA for 48h exposure time compared with NFs. Although the effect of 0.55% DMSO increased collagen production in NFs, the
production caused by 0.55% DMSO did not show specific significance in KFs. Concurrently, after TA treatment, staining signals of intracellular and extracellular were decreased. Stained collagen secreted to extracellular was decreased with cytoplasm concentrated and thus cell density was decreased. Therefore, these outcomes indicated that TA reduced collagen production in KFs with high efficiency. This also further pointed out that the inhibitory effect of TA on collagen accumulation is associated with inducing cell apoptosis \textit{in vitro}.

\textit{Tolfenamic Acid Significantly Inhibited the Expression of Collagen Type I in Human Keloid Fibroblasts}

In collagen quantification assay, the type of stained collagen is non-specific. As studies showed the majority of collagen produced by KFs is collagen type I, we particularly test the expression of collagen type I after TA treatment \cite{1, 2, 16, 17, 19, 20}. Our study showed that there is a remarkably increasing expression of collagen I in KFs compared with NFs. Moreover, 0.55x10^{-3} M TA for 48h exposure time significantly inhibited the expression of collagen type I in KFs compared with NFs \textit{in vitro}. 0.55% DMSO group did not show significant inhibitions of the expression of collagen type I in NFs and KFs. This revealed that DMSO did not significantly influence the expression of collagen type I and the inhibitory effect of TA on collagen accumulation, especially collagen type I, is associated with inducing cell apoptosis \textit{in vitro}.

\textit{Tolfenamic Acid Inhibited the Expression of Smad2 Protein in Both}
**Human Normal Fibroblasts and Keloid Fibroblasts**

TGF-β is a key cytokine of regulating extracellular and intracellular signaling pathway. Current studies about TGF-β signaling pathway downstream transduction in KFs suggested that smad family was the fundamental mediator influencing nuclear gene expression [16, 19, 20]. The amount of smad family accumulated in the nucleus and the duration was coordinated with their target promoters for the transcriptional responses. Smad2 is a critical cytokine in regulating collagen gene expression which will cause pathogenically fibroproliferation during keloid formation [19, 20]. Blocking Smad2 signaling pathway could theoretically suppress the production of collagen, thus preventing the occurrence of keloid scars. This also implied an accessible way in eliminating or blocking the accumulation of collagen in treating KFs. In our study, 0.55% DMSO for 48h inhibits smad2 expression in NFs. By combining our data together, it suggested that DMSO was able to affect NF activities in cell apoptosis and collagen production while the influence in KFs was not significant compared with TA treatment. Also, our study illustrated that 0.55x10^{-3}M TA for 48h inhibits the expression of smad2 protein in both NFs and KFs. This signifies that inhibitory effect of TA on smad2 expression in NFs and KFs. To our knowledge, there is increased signaling and elevated levels of smad complexes in keloid scar, which substantiates the possible model of an autocrine positive feedback loop in keloid scar [12,13, 15, 17, 19, 21]. Due to the hyperactivity of KF, cell activities such as cell apoptosis and collagen production especially collagen type I were significantly increased comparing with NF. However, the expression of smad2 protein in both NFs and KFs were
inhibited. This possibly suggested that in this keloid sample the expression of smad2 protein was undercontrolled by the autocrine positive feedback loop. Although there is an over production of collagen type I, the autocrine positive feedback loop is regulated by the smad2 signaling transduction in TGF-β/Smad signaling pathway in KFs. TA can break the autocrine positive feedback in NFs and lead the expression of smad2 protein reduced. But, with the hyperactivity of TGF-β/Smad signaling and the regulation of autocrine positive feedback loop, the inhibition in KFs after TA is not that significant compared with NFs. The activity of smad2 and autocrine positive feedback loop in KFs expected to conduct in further studies.

**The Outlook of Tolfenamic Acid on Treating Keloid Fibroblasts**

For further studies, other protein expressions of collagen especially type III collagen, smad family treated after TA within more samples, and autocrine positive feedback loop in KFs are taken into consideration. At the same time, agonists, antagonists and signaling blockers of TGF-β signaling pathway, for example, TGF-β receptor I blockers, TGF-β receptor II blockers, phosphorylated smad family are expected to further confirm the mechanism of TA on KFs in the future. In the cell signaling pathway of TGF-β/Smad, specific protein 1 is discovered as the transcription factor that influences the DNA binding or DNA associated protein binding for nuclear gene expression. The transcription for specific protein 1 is activated by phosphorylated smad family. Additionally, the established data showed that specific protein 1 is a new targeting approach of transcription factors for keloid scar. TA could also induce
apoptosis in cancer cell via being associated with inhibiting specific protein 1 activities [22, 42, 45, 46]. Hence, specific protein 1 is one cytokine of the smad signaling pathway downstream that potentially affected protein expression in cellular response. This intriguing idea can be inserted into study the mechanism of TA on KFs.
VII. CONCLUSION

Generally speaking, our current novel data demonstrated that:

1) 1% DMSO with 48h exposure time did not significantly induce cell apoptosis in NFs and KFs;

2) $10^{-3}$M TA strikingly decreased cell proliferation in NFs and KFs;

3) $0.55 \times 10^{-3}$M TA selectively induced cell apoptosis in KFs;

4) $0.55 \times 10^{-3}$M TA obviously reduced collagen production in KFs;

5) $0.55 \times 10^{-3}$M TA significantly inhibited the expression of collagen type I in KFs;

6) $0.55 \times 10^{-3}$M TA inhibited the expression of smad2 protein in both NFs and KFs.

Therefore, we illustrated that tolfenamic acid induced cell apoptosis and inhibited collagen production in keloid fibroblast. TA could be the new therapeutically application for treating keloid scars. With the development of the advanced Technologies, hundreds and thousands of treatments on keloid scar have been reported. However, this is the first time where TA was successfully used in vitro to induce cell apoptosis and reduce collagen accumulation in KFs. Furthermore, TA is an available commercial formulation chemical. Thereby, TA is recommended for clinical trials to confirm our findings.
VIII. REFERENCES


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