

Original Article

Dehydroabietic acid reverses TNF- α -induced the activation of FOXO1 and suppression of TGF- β 1/Smad signaling in human adult dermal fibroblasts

Xiao-Wei Wang^{1*}, Yong Yu^{2*}, Lei Gu³

¹Department of Endocrinology, Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University, Shanghai 201707, People's Republic of China; ²Key Laboratory of Viral Heart Disease Ministry of Public Health, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital Fudan University, Shanghai 20032, People's Republic of China; ³Department of Emergency, Shanghai Tongji Hospital, Shanghai 200065, People's Republic of China. *Equal contributors.

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Abstract: Wound healing impairment is a well-documented phenomenon in clinical and experimental diabetes, and in diabetic wound healing impaired fibroblast has been linked to increased levels of tumor necrosis factor- α (TNF- α). A number of signaling pathways including TNF- α /forkhead box O1 (FOXO1) and transforming growth factor- β 1 (TGF- β 1)/Smads in fibroblasts appear to play a cardinal role in diabetic wound healing. Dehydroabietic acid (DAA) is obtained from *Commiphora oppbalsamum* and inhibits the production of TNF- α in macrophages and adipocytes, decreases the level of TNF- α in obese diabetic KK-Ay mice, but its effect on diabetic wound healing is unknown. This study was to investigate the effect of DAA on TNF- α -stimulated human adult dermal fibroblasts. On the one hand, TNF- α significantly decreased the fibroblast proliferation and the expression of PCNA, Ki67 and cyclin D1, increased the fibroblast apoptosis, caspase-8/3 activity, expressions of cleaved caspase-8 and caspase-3, decreased the Bcl-2/Bax ratio and increased activation of the pro-apoptotic transcription factor FOXO1. All the above-mentioned cell responses were remarkably reversed by DAA. On the other hand, TNF- α also inhibited TGF- β 1-induced the Smad3 signaling pathway what is closely related to the fibroblast migration and the differentiation of myofibroblasts. However, DAA significantly promoted the migration and increased the expression of α -smooth muscle actin and fibronectin under the stimulus of a combination of TNF- α and TGF- β 1. In conclusion, DAA could reverse several cell responses stimulated by TNF- α , including the activation of FOXO1 and the TGF- β 1/Smad3 signaling pathway. These results suggested that DAA could be useful in improving the diabetic wound healing.

Keywords: Dehydroabietic acid, tumor necrosis factor- α , transforming growth factor- β 1, forkhead box o1, diabetes, wound healing

Introduction

In diabetes, impaired wound healing is a serious complication, which can result in limb amputation [1]. Once a wound happens, many diverse types of cells are recruited to take part in the wound repair process [2]. Fibroblasts are the most usual cells that are contained in connective tissue, and play an important role in wound healing. Fibroblasts are activated and then proliferate and migrate to the wound site [3]. Moreover, fibroblasts are differentiated into a specific transiently expressed phenotype known as myofibroblasts [4]. However, it has been

reported that the mechanism of diabetic wound healing is associated with the increase of fibroblast apoptosis [5].

It has been reported that inflammatory factors are increased in diabetic wounds and may induce apoptosis through activation of the pro-apoptotic transcription factor, forkhead box O1 (FOXO1), and activation of caspase-3 [5]. Tumor necrosis factor- α (TNF- α) is a potent pro-inflammatory and pro-apoptotic mediator that plays a critical role in normal and diabetic wound healing [6]. A potential mechanism through which diabetes may increase apoptosis is the over-

much production of the TNF- α [7]. The levels of TNF- α are increased in non-healing ulcers [8] and closely related to impaired healing in diabetic db/db mice [7].

The inhibition of fibroblast proliferation has been related to the enhancement of TNF- α level in diabetic wound healing [9]. Fibroblasts apoptosis of diabetic mice is dramatically higher than that of normoglycemic counterparts [7], and apoptosis is also high in skin biopsies from diabetic foot ulcers [10]. Previous studies showed that diabetes increased the levels of TNF- α , inhibited fibroblast proliferation, induced apoptosis of fibroblast, and stimulated activation of the FOXO1. Once TNF- α is blocked, there is improved fibroblast proliferation, decreased apoptosis, higher fibroblast density, leading to improve wound healing of the diabetic group [7].

Recent studies also have demonstrated that TNF- α may have antagonistic activities against TGF- β 1. The Smad pathway constitutes a family of serine-threonine kinase proteins involved in TGF- β signaling that plays a critical role in the induction of myofibroblastic differentiation and fibroblasts migration under the stimulus of TGF- β 1 [11, 12]. In dermal fibroblasts, TNF- α inhibited the phosphorylation of Smad2/3 with stimulus of TGF- β 1 [13], resulting in suppression of myofibroblastic differentiation. Moreover, high levels of TNF- α restrain fibroblasts migration, which may proceed by enhancing the level of Smad 7 and reducing the phosphorylation of Smad 2 [12].

Dehydroabietic acid (DAA), a diterpene, is obtained from *Commiphora oppbalsamum*. Terpenoids that are contained in numerous dietary and herbs have extensive biological effects. It has been reported that DAA inhibits the production of proinflammatory mediators such as TNF- α in macrophages and adipocytes [14], decreases the level of TNF- α in obese diabetic KK-Ay mice and is considered as a food-derived and valuable medicinal compound for ameliorating the inflammation caused by obesity of diabetes [15].

However, the effects of DAA on impaired wound healing of diabetes were still unknown. Therefore, in the present study, to investigate the effects of DAA on diabetic wound, we examined the effects and mechanisms of DAA on TNF- α -induced fibroblasts apoptosis, inhibition of myofibroblastic differentiation and fibroblasts migration.

Materials and methods

Cell culture and treatment

Primary human adult dermal fibroblasts (HADF) were purchased from Cambrex (Walkersville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco, New York, USA) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal calf serum (FBS) (Gibco, New York, USA) at 37°C in 5% CO₂ on 0.1% gelatin-coated culture flasks. Passage 3-8 HADFs were used for experiments. DAA is purchased from Sigma (USA, CAS No. 1740-19-8). HADFs were treated with TGF- β 1 (20 ng/ml) or TNF- α (20 ng/ml) and combinations of DAA (5, 10, 20, 40 μ M or 40 μ M) and TNF- α (20 ng/ml) with or without TGF- β 1 (20 ng/ml) for 30 min, 24 h, 48 h or 72 h in DMEM containing 0.1% FBS. This cell culture condition was used in our test analysis.

Cell proliferation assay

HADFs were plated at 1×10^4 cells/well in 96 well culture plates. After incubation with test medium for 24 h, the number of viable cells was determined using the mitochondrial tetrazolium (MTT) reagent according to the manufacturer's instructions.

To investigate the effect of DAA on HADFs cell proliferation, 1×10^4 cells were treated with the test medium for 24 h at 37°C. Cell Proliferation ELISA-BrdU (colorimetric) Kit (Roche Diagnostics, USA) was used to determine the cells proliferation according to the manufacturer's instructions. The DAA concentration with the highest proliferating effect was used for other experiments.

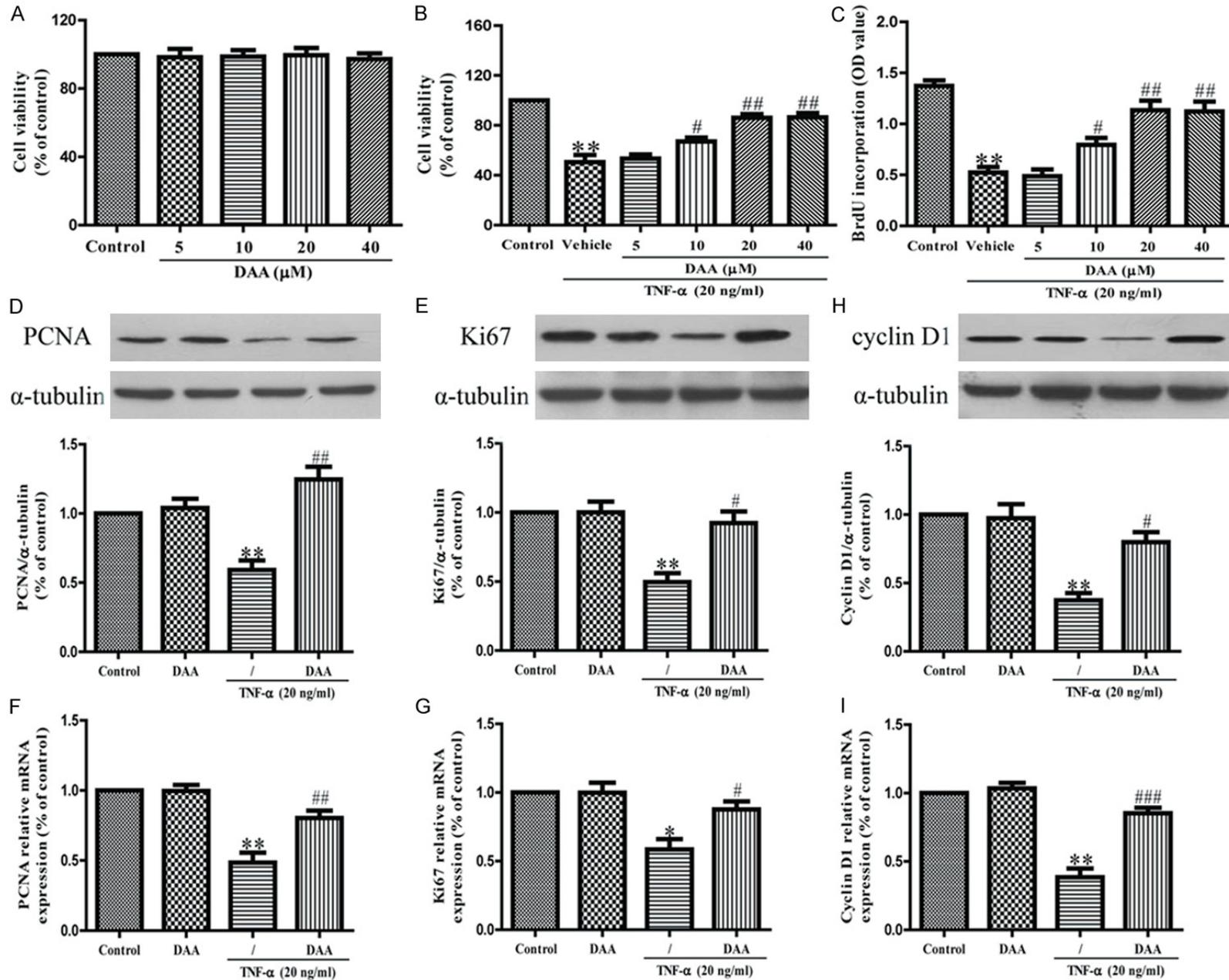
Detection of apoptosis

Apoptosis was determined by using Cell Death ELISA Detection Kit (Switzerland; Roche, Inc.) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation. Cell apoptosis detection was performed under the manufacturer's instructions and monitored spectrometrically at 405 nm.

Cell migration assay

Cell migration assay was performed using Transwell chambers. The bottom chamber of the apparatus contained test medium. The HADFs were added to the upper chamber. After 12 h

Dehydroabietic acid and human adult dermal fibroblasts



Dehydroabietic acid and human adult dermal fibroblasts

Figure 1. Effect of DAA on the cell viability and proliferation of HADFs under stimulus of TNF- α . HADFs were treated with vehicle, TNF- α (20 ng/ml), or combinations of DAA (5, 10, 20, 40 μ M or 40 μ M) and TNF- α (20 ng/ml) for 24 h. A, B. Cell viability was estimated by MTT assay. C. Cell proliferation was evaluated by BrdU-ELISA assay. D, E. Protein levels of PCNA and Ki67 were detected by Western Blot using α -tubulin as an internal control. F, G. PCNA mRNA and Ki67 mRNA were determined by RT-PCR. H, I. Protein expression and mRNA level of cyclin D1 were measured by RT-PCR and Western Blot, respectively. Result represented as proportion of control (100%). All data are presented as mean \pm SEM, n = 6. * P < 0.05, ** P < 0.01 vs. control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. TNF- α group.

incubation at 37°C, non-invasive cells on the upper membrane surfaces were removed by wiping with cotton swabs. Invading cells were fixed with methanol and stained with 0.1% crystal violet. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscopy. Then the membranes were washed with 30% glacial acetic acid. For HADFs counting determination, the washing solution was detected at the wavelength of 540 nm.

Reverse transcription polymerase chain reaction

Total RNA of HADFs was extracted by using Trizol reagent (Life Technologies, Carlsbad, CA). Two microgram RNA was used for gene-specific reverse transcription polymerase chain reaction (RT-PCR) using one-step RT-PCR kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. The following primers were used: PCNA forward primer: 5'-AGA-AGGTGTTGGAGGCACTCA-3'; reverse primer: 5'-GGTTTACACCGCTGGAGCTAA-3'; Ki67 forward primer: 5'-AACTATCCTCGTCTGTCCCAACAC-3'; reverse primer: 5'-CGGCCATTGGAAAGACAGAT-3'; cyclin D1 forward primer: (5'-TGAGAGAAA-AAGGCCTACG-3'); reverse primer: 5'-GTAGCAG-CTACTGTAGACAG-3'); α -SMA forward primer: 5'-CCGACCGAATGCAGAAGGA-3'; reverse primer: 5'-ACAGAGTATTTGCGCTCCGAA-3'; collagen I forward primer: 5'-CAGCCGCTTCACTACAGC-3'; reverse primer: 5'-TTTTGTATTCAACTGTCTTGCC-3'; fibronectin forward primer: 5'-GGAGAA-TTCAAGTGTGACCCTCA-3'; reverse primer: 5'-TG-CCACTGTTCTCTACGTGG-3'; HSP-47 forward primer: 5'-CGCCATGTTCTTCAAGCCA-3'; reverse primer: 5'-CATGAAGCCACGGTTGTCC-3'; ED-A-FN forward primer: 5'-CCAGTCCACAGCTATTC-CTG-3'; reverse primer: 5'-ACAACCACGGATGAG-CTG-3'; GAPDH forward primer: 5'-GAAGGTGA-AGGTCGGAGT-3'; reverse primer: 5'-GAAGATGG-TGATGGGATTTC-3'.

The levels for each gene were counted by standardizing the quantified mRNA amount to the GAPDH mRNA. Each sample was assessed in triplicate.

Western blot analysis

HADFs were lysed using protein lysis buffer and protease inhibitor cocktail. Equal amounts of protein were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were incubated with the following primary antibodies: phospho-Smad3, Smad3, phospho-FoxO1, FoxO1, Cleaved caspase 8, Cleaved caspase 3, cyclin D1, Bcl-2, Bax (1:1000; CST), PCNA, Ki67, HSP47, Fibronectin (1:1000; Santa Cruz), ED-A-FN (1:1000; Sigma) or α -SMA (1:1000; Beyotime Institute of Biotechnology, China), and then incubated with an HRP-conjugated secondary antibody. Incubation with anti- β -actin antibody (1:1000; CST) or anti- α -tubulin antibody (1:1000; Sigma) was performed as a control. The proteins were visualized using ECL kit.

Caspase-8/3 activity assay

Caspase activity was determined by caspase-8 or 3 fluorescent assay kit (NanJing KeyGen Biotech, China) according to the manufacture's guidance. In brief, HADFs were treated with test medium. Cells were lysed in the lysis buffer and centrifuged, and then the supernatants were gathered. The reactions that reacted equal quantities of protein samples with the synthetic fluorescent substrates at 37°C for 1.5 h were read at 405 nm in a microplate reader, and bovine serum albumin (BSA) was considered as the control. Fold-increases in caspase-8 and 3 activities were detected with quantitative values gained from the treatment samples separated by those from the controls.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Data for each study parameter from each group were presented as mean \pm standard error of the mean (S.E.M.). Data from each group were statistically analyzed by a two-tailed Student's t test or one-way analysis of variance

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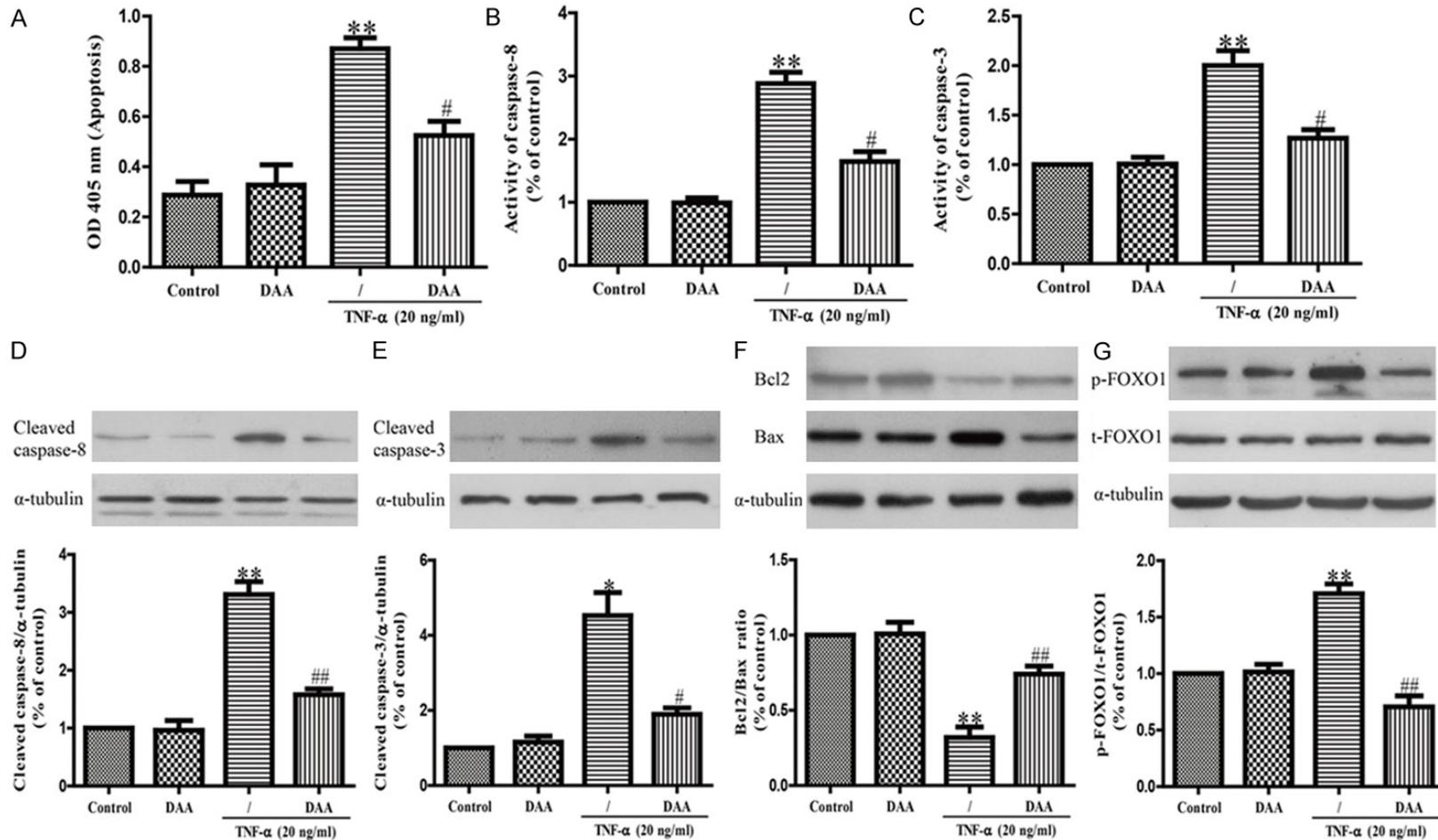


Figure 2. Effects of DAA on TNF- α -induced apoptosis of HADFs and activation of FOXO1. HADFs were treated with vehicle, DAA (40 μ M) or TNF- α (20 ng/ml) with or without DAA (40 μ M) for 24 h or 30 min. A. Apoptosis of HADFs was measured by nucleosomal degradation by using Roche's cell death ELISA detection kit. B, C. Activities of caspase-8 and caspase-3 were determined by caspase-8 and caspase-3 activities detection assay. D, E. Protein levels of cleaved caspase-8 and caspase-3 were detected by Western Blot using α -tubulin as an internal control. F. Protein expressions of Bcl-2 and Bax were detected by Western Blot using α -tubulin as an internal control. G. Activation of FOXO1 was studied by estimating phosphorylation of FOXO1 that was determined by Western Blot. All data are presented as mean \pm SEM, n = 6. * P < 0.05, ** P < 0.01 vs. control; # P < 0.05, ## P < 0.01 vs. TNF- α group.

(ANOVA). Differences were considered statistically significant at $P < 0.05$.

Results

DAA attenuated the TNF- α -induced decrease of cell viability and proliferation of HADFs

HADFs were treated with different concentrations of DAA (5, 10, 20 and 40 μM) for 24 h in the absence of TNF- α , and the cell viability was evaluated by MTT assay (**Figure 1A**). There were no effects of DAA on the growth of HADFs. When HADFs were treated with TNF- α (20 ng/ml), the cell viability was significantly decreased compared with control group (**Figure 1B**). However, DAA reversed the inhibition of cell viability induced by TNF- α in a concentration-dependent manner with a maximal effect at 20 and 40 μM . Similarly, results by BrdU cell proliferation assay were in accordance with those of MTT assay (**Figure 1C**). From above results, the concentration of 40 μM was used in the following experiments.

To further confirm these results, the expressions of PCNA and Ki67 that are two proliferation markers were detected by Western blot analysis. As shown in **Figure 1D** and **1E**, treatment of HADFs with TNF- α for 24 h dramatically decreased PCNA and Ki67 protein expressions in comparison to the control group, but both of them were significantly enhanced by DAA at 40 μM . Accordingly, the mRNA levels of PCNA and Ki67 were decreased by TNF- α treatment but increased by DAA at 40 μM (**Figure 1F, 1G**). Besides, cyclin D1 is a nuclear protein required for cell cycle regulation in the G1 phase of proliferating cells, while its mRNA level and protein expression were down-regulated in the presence of TNF- α , DAA could remarkably attenuated the TNF- α -induced the down-regulation of cyclin D1 (**Figure 1H, 1I**).

DAA inhibited TNF- α -induced apoptosis of HADFs and activation of FOXO1

To confirm whether DAA improved TNF- α -mediated growth arrest by inhibiting apoptosis, we detected nucleosomal degradation as well as caspase-8 and caspase-3 activation under the same conditions used in the above experiments. The results shown in **Figure 2A-C** demonstrated that treatment of HADFs with TNF- α (20 ng/ml) induced nucleosomal degradation,

caspase-8 and caspase-3 activation. Treatment of HADFs with DAA and TNF- α attenuated these alterations. To further confirm these results, the expressions of cleaved caspase-8 and caspase-3 were detected by Western blot analysis (**Figure 2D, 2E**). Treatment of HADFs with TNF- α increased the expressions of cleaved caspase-8 and caspase-3 proteins, which are critical effector caspases that induce apoptosis. However, DAA treatment significantly reduced the expressions of both the above-mentioned proteins. In addition, TNF- α also decreased Bcl-2 and increased Bax protein expressions in comparison to the control group, which resulted in a low ratio of Bcl-2/Bax. The balance between Bcl-2 and Bax is important in activating and deactivating the downstream cellular apoptosis pathway [16]. We found that DAA could improve the ratio of Bcl-2/Bax to inhibit the apoptosis of fibroblasts stimulated by TNF- α (**Figure 2F**).

It has been previously shown that TNF- α promotes fibroblasts apoptosis by stimulating the transcription factor, FOXO1 [17]. To better understand the impact of DAA on preventing fibroblasts from apoptosis induced by TNF- α , the activation of FOXO1 was studied (**Figure 2G**). The results showed that DAA had no effect on the phosphorylation of FOXO1, which was the same as the control group. There were statistically significant differences in FOXO1 phosphorylation between TNF- α group and control group. However, DAA could dramatically reverse the up-regulation of FOXO1 phosphorylation induced by TNF- α .

DAA improved down-regulation of the production of TGF- β 1-stimulated extracellular matrix (ECM) molecules induced by TNF- α

To identify the effects of DAA on the production of TGF- β 1-stimulated (20 ng/ml) ECM molecules induced by TNF- α (20 ng/ml). Protein expressions for type I collagen (**Figure 3A**), HSP-47 (**Figure 3B**) and fibronectin (FN) (**Figure 3C**) were estimated by Western blot analysis of the cell lysates. As shown in **Figure 3**, TGF- β 1 significantly enhanced the protein expressions of all these ECM molecules. Costimulation with TGF- β 1 and TNF- α dramatically down-regulated the protein levels of all these molecules. However, DAA markedly improved down-regulation of the production of TGF- β 1-stimulated ECM molecules induced by TNF- α . Moreover, quantification and analysis of mRNA levels showed

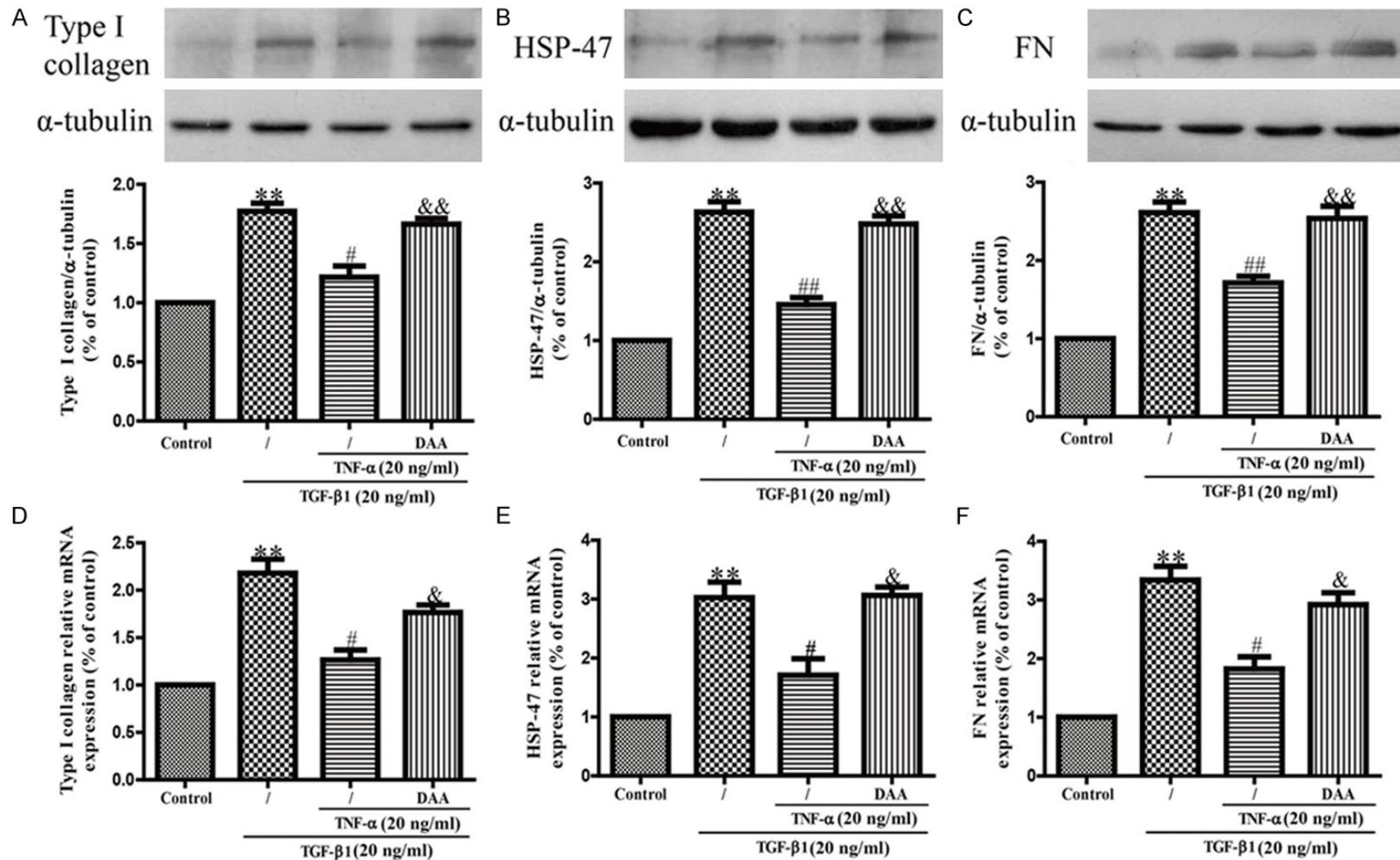


Figure 3. Effect of DAA on regulation of production of TGF- β 1-stimulated ECM molecules induced by TNF- α . HADFs were treated with vehicle, TNF- α (20 ng/ml), TGF- β 1 (20 ng/ml), or combinations of TGF- β 1 (20 ng/ml) and TNF- α (20 ng/ml) with or without DAA (40 μ M) for 48 h or 24 h in DMEM containing 0.1% FBS. Protein expressions of type I collagen (A), HSP-47 (B) and FN (C) were detected by Western Blot using β -actin as an internal control. The mRNA levels of type I collagen (D), HSP-47 (E) and FN (F) were determined by RT-PCR. All data are presented as mean \pm SEM, n = 6. ***P* < 0.01 vs. control; #*P* < 0.05, ##*P* < 0.01 vs. TGF- β 1 group; &*P* < 0.05, &&*P* < 0.01 vs. TGF- β 1 + TNF- α group.

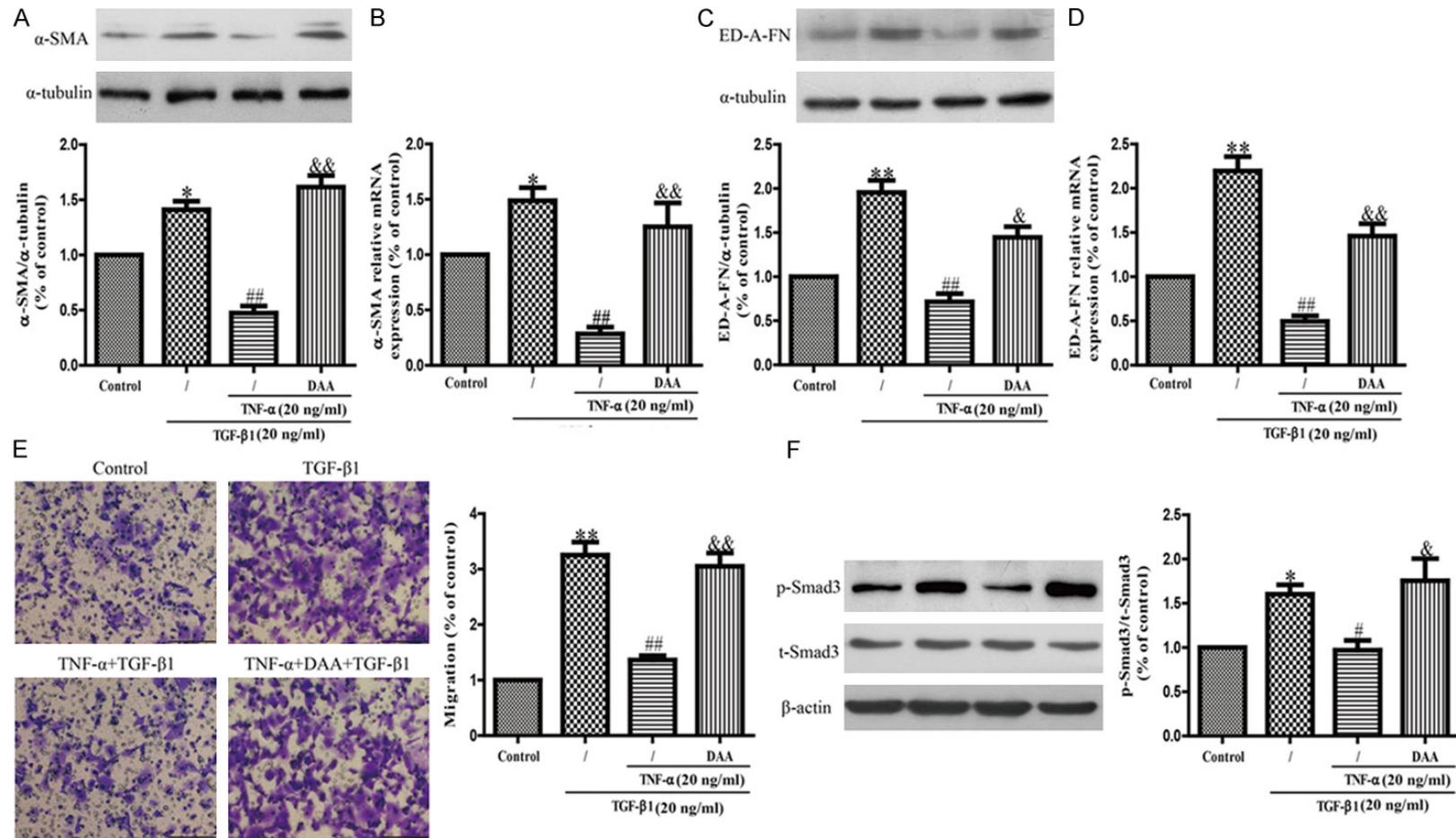


Figure 4. Effects of DAA on production of α-SMA and ED-A-FN, cell migration and the Smad pathway stimulated by TGF-β1 combined with TNF-α. HADFs were treated with TNF-α (20 ng/ml), TGF-β1 (20 ng/ml), or combinations of TGF-β1 (20 ng/ml) and TNF-α (20 ng/ml) with or without DAA (40 μM) for 30 min, 24 h or 72 h in DMEM containing 0.1% FBS. A, B. Protein levels of α-SMA and ED-A-FN were detected by Western Blot using α-tubulin as an internal control. C, D. α-SMA mRNA and ED-A-FN mRNA were determined by RT-PCR. E. Cell migration of HADFs was measured by Transwell assay. F. Phosphorylation of Smad3 was determined by Western Blot using β-actin as an internal control. All data are presented as mean ± SEM, n = 6. **P* < 0.05, ***P* < 0.01 vs. control; #*P* < 0.05, ##*P* < 0.01 vs. TGF-β1 group; &*P* < 0.05, &&*P* < 0.01 vs. TGF-β1 + TNF-α group.

that DAA increased the production of TGF- β 1-stimulated type I collagen, HSP-47 and FN at statistically significant levels in the presence of TNF- α (**Figure 3D-F**), which were similar with the results of the Western blot analysis.

DAA enhanced TGF- β 1-stimulated myofibroblastic differentiation and promoted cell migration through the Smad pathway in the presence of TNF- α

To examine whether DAA exhibited the antagonistic activity against the ability of TNF- α (20 ng/ml) to downregulate the level of α -SMA stimulated by TGF- β 1 (20 ng/ml), the protein expressions of α -SMA that is a character of myofibroblasts were analyzed by Western blot analysis of the cell lysates (**Figure 4A**). TGF- β 1 remarkably induced α -SMA production. It was very interesting that TNF- α reversed the ability of TGF- β 1 to upregulate the expression of α -SMA during costimulation with TNF- α and TGF- β 1. DAA markedly improved down-regulation of the production of TGF- β 1-stimulated α -SMA induced by TNF- α . We then estimated the regulation of ED-A-FN, an isoform of FN, which also plays a critical role in myofibroblastic differentiation. Eventually, we found that results of ED-A-FN were consistent with those results of α -SMA (**Figure 4B**). Moreover, mRNA levels of α -SMA and ED-A-FN were determined by using RT-PCR. We showed that TGF- β 1 induced increases in α -SMA (**Figure 4C**) and ED-A-FN (**Figure 4D**) mRNA levels after 24 hours of cytokine treatments. However, when fibroblasts were treated with TNF- α plus TGF- β 1, the mRNA levels of both genes were suppressed. Treatment with DAA, the suppression of both mRNA levels was significantly improved.

Migration of fibroblasts also plays an essential role in wound repair. Cell migration assay was performed using Transwell chambers (**Figure 4E**). The results showed that the number of migrated fibroblasts was much more than those of control group. Interestingly, the mobility of TGF- β 1-stimulated fibroblasts was obviously inhibited by TNF- α , and then DAA distinctly restored the mobility of fibroblasts treated with TGF- β 1 combined with TNF- α .

Smad signaling is considered the principal signaling pathway for TGF- β , which is also responsible for differentiation of myofibroblasts and migration of fibroblasts [3, 4]. Subsequently,

we analyzed the phosphorylation of Smad3 under the stimulus of TGF- β 1, and a combination of both cytokines with or without DAA. As shown in **Figure 4F**, TGF- β 1 activated phosphorylation of Smad3 after 30 minutes of stimulation. In contrast, TNF- α suppressed phosphorylation of Smad3 induced by TGF- β 1. However, DAA visibly improved the inhibition of Smad3 phosphorylation treated with TGF- β 1 combined with TNF- α . Therefore, it is possibly that DAA restored TNF- α suppression of TGF- β 1-induced α -SMA expression and fibroblast migration by promoting Smad3 phosphorylation.

Discussion

Previous studies had reported that TNF- α , a proinflammatory cytokines, was elevated in both type I and type II diabetic wounds. Moreover, DNA activity of FOXO1 that is a pro-apoptotic transcription factor was increased in diabetic wounds, and the activation of FOXO1 was dramatically mediated by diabetes-enhanced TNF- α level, leading to higher levels of apoptosis and inhibition of fibroblasts proliferation. While TNF- α was specifically blocked, healing of both type I and type II diabetic wounds was promoted [7].

Terpenoids, which are contained in most of dietary and herbal plants, exert many biological activities. In this research, the effects of DAA, a diterpene, on HADFs treated with TNF- α combined with or without TGF- β 1 were investigated. We showed here that DAA treatment not only attenuated the TNF- α -induced decrease of proliferation of HADFs and inhibited apoptosis of HADFs induced by TNF- α , but also improved the inhibition of TGF- β 1-stimulated differentiation of myofibroblasts and migration of fibroblasts induced by TNF- α . To examine the mechanism underlying the effects of DAA, the phosphorylation of FOXO1 and Smad3 was measured. It was shown that the DAA treatment significantly suppressed the TNF- α -activated phosphorylation of FOXO1 and improved the inhibition of Smad3 phosphorylation treated with TGF- β 1 combined with TNF- α .

Fibroblast proliferation is a critical stage in wound healing. Importantly, not only MTT assay and BrdU assay but also the detection of Ki67, PCNA and cyclin D1 expression, which are essential for DNA replication, were used to investigate the effect of DAA on TNF- α -induced

decrease of HADFs proliferation in this study. We confirmed that DAA attenuated the TNF- α -stimulated inhibition of HADFs proliferation. It has been reported that TNF- α induced the apoptosis of vascular adventitial fibroblasts (VAFs) by decreasing the ratio of Bcl-2/Bax. Moreover, decrease in Bcl-2/Bax ratio leads to a change in the $\Delta\Psi_m$, resulting in activating effectors caspase-3, and subsequently inducing cell apoptosis. However, knockdown of FOXO1 significantly inhibited apoptosis of VAFs stimulated with TNF- α , which suggested that FOXO1 could regulate the expressions of target genes including Bcl-2, Bax and caspase-3, and then promote TNF- α -induced VAFs apoptosis [18]. As is well known, FOXO1 regulates cell apoptosis by phosphorylation, acetylation and ubiquitination modifications [19]. We investigated whether the DAA protected the fibroblasts from TNF- α -induced apoptosis by inhibiting phosphorylation of FOXO1. Data from this study suggested that DAA inhibited TNF- α -induced HADFs apoptosis by suppressing phosphorylation of FOXO1, thereby increasing Bcl-2/Bax ratio and deactivate caspase-8 and -3, decreasing the cleaved caspase-8 and caspase-3 protein expression.

Cell migration and cellular phenotypic alterations inside the wound environment play a critical role in response to injury [3, 13]. The granulation tissue formation of the wound site needs neighbor fibroblasts to proliferate and migrate to the wound site, which is notably considerable when fibroblasts migrate to wound site to transform into myfibroblasts and produce ECM proteins including type I collagen, HSP-47 and FN in formation of granulation tissue. These activated fibroblasts or myfibroblasts is major characterized by the expression of α -SMA. Besides, ED-A-FN is a spliced variant of FN that is associated with myfibroblastic differentiation [11]. Actually, ED-A-FN is very crucial because it enhances the expression of α -SMA [20]. Therefore, reduction of ED-A-FN production may reflect an inchoate incident in the suppression of myfibroblastic differentiation induced by TNF- α . Myfibroblasts are special fibroblasts that conduce to wound healing by producing ECM and by bringing contractility to hold the edges of a wound together. ECM is the considerable ingredients of granulation tissues, which supply a frame for a number of wound healing processes.

TGF- β 1 activates the phosphorylation of the Smad proteins that plays an important role in

regulating the expression of several TGF- β -regulated ECM, such as type I collagen, HSP-47 and FN [11]. In diabetic wound healing, it has been exhibited that TNF- α can develop an antagonistic effect on a few responses stimulated or induced by TGF- β 1. The delayed healing in diabetic wound revealed that the increase of TNF- α level evidently suppressed TGF- β 1-induced myfibroblastic differentiation and fibroblasts migration. Additionally, previous reports also have determined that TNF- α can reduce TGF- β 1-induced type I collagen, HSP-47 and FN production in dermal fibroblasts [13], and our present results coincided with these results. In this study, DAA reversed the TNF- α -induced inhibition of the phosphorylation of Smad3 under TGF- β 1 stimulus.

In summary, the present study showed that DAA could reverse several cell responses stimulated by TNF- α . Firstly, DAA inhibited TNF- α -induced apoptosis of HADFs by deactivation of FOXO1. Secondly, DAA enhanced TNF- α -inhibited the ECM production, myfibroblastic differentiation and migration of HADFs by improving the TGF- β 1/Smad3 signaling pathway. These results suggested that DAA could be useful in improving the diabetic wound healing.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiao-Wei Wang, Department of Endocrinology, Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University, Shanghai 201707, People's Republic of China. Tel: +86 21 86574006; Fax: +86 21 86574006; E-mail: wangxiaoweibmb@163.com; Dr. Lei Gu, Department of Emergency, Shanghai Tongji Hospital, Shanghai 200065, People's Republic of China. Tel: +86 21 84772352; Fax: +86 21 84772352; E-mail: hebenmedmail@163.com

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