Increased sensitivity to TNF-α promotes keloid fibroblast hyperproliferation by activating the NF-κB, JNK and p38 MAPK pathways

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Received August 19, 2020; Accepted January 18, 2021

DOI: 10.3892/etm.2021.9933

Abstract. Hyperproliferation of fibroblasts is the main cause of keloid formation. However, the pathogenesis of keloids has yet to be fully elucidated. Tumor necrosis factor (TNF)-α may play an important role in the formation and proliferation of keloids, as it is implicated in the pathogenesis of various fibrous disorders. In the present study, the expression level of TNF-α and its receptors, soluble TNF receptor (sTNFR)1 and sTNFR2, in the peripheral blood and skin tissues was detected by ELISA, reverse transcription-quantitative PCR or immunohistochemistry. There was no statistically significant difference in the expression of TNF-α and sTNFR2 in the peripheral blood and skin tissues between patients with keloids and healthy participants (P>0.05), while the sTNFR1 mRNA level in fibroblasts cultured *in vitro* and its protein level in keloid skin samples were significantly higher compared with those in normal skin (P<0.05). Subsequently, TNF-α recombinant protein was used to treat keloid-derived and normal skin fibroblasts, and it was observed that TNF-α promoted the proliferation of keloid fibroblasts (KFs), but had little effect on normal skin fibroblasts. Furthermore, it was observed that TNF-α stimulation led to the activation of the nuclear factor (NF)-κB, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways in KFs. In conclusion, KFs exhibited increased expression of sTNFR1, which may contribute to the increased sensitivity to TNF-α, resulting in low concentrations of TNF-α activating the NF-κB, JNK and p38 MAPK pathways, thereby promoting the sustained and excessive proliferation of KFs.

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Key words: keloid fibroblasts, tumor necrosis factor-α, proliferation, sensitivity, tumor necrosis factor receptor 1

Introduction

Keloid is a type of proliferative fibroma that may form after trauma, and is characterized by abnormal wound healing, fibroblast proliferation and extracellular matrix deposition. Keloid is considered as a benign human tumor without malignant potential, which invades the adjacent normal skin tissue beyond the initial boundaries of the trauma (1,2). Fibroblasts are considered to play a key role in the formation and development of keloids (3). The hyperproliferation of fibroblasts and deposition of extracellular matrix results in collagen metabolism disorder, which is the main cause of keloid fibrosis. However, the pathogenesis of keloids has not been fully elucidated (4,5).

Researchers have found that keloids are accompanied by chronic inflammation and immune abnormalities (5), suggesting that proinflammatory cytokines may be involved in the keloid formation process. Tumor necrosis factor (TNF)-α is a type of cytokine, which is secreted by various cells and is involved in the maintenance and homeostasis of the immune system, inflammation and host defense. However, TNF-α is also involved in pathological processes such as chronic inflammation, autoimmunity and malignant diseases (6). In particular, TNF-α is involved in the pathogenesis of various fibrotic disorders, including liver fibrosis, pulmonary fibrosis, cardiac fibrosis and scleroderma, among others (7). The level of serum TNF-α in African American individuals with keloids was observed to be higher compared with that in normal subjects without keloids (8), and the expression of TNF-α in the keloid was shown to be higher compared with that in normal tissues (9). Zhu *et al* (10) found that stimulation with TNF-α at a low concentration (50 ng/ml) may lead to the proliferation of keloid fibroblasts (KFs) *in vitro*. However, the specific role and mechanism of

action of TNF-α in the pathogenesis of keloids are unclear. In tumor research, TNF-α was found to promote cell proliferation through activating the nuclear factor (NF)-κB, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, which were regulated by the concentration of TNF-α (11). However, further experimental research is required to determine whether the function and mechanism of action of TNF-α in tumors is involved in keloid pathogenesis.

Based on the keloid characteristics and the role of TNF-α in fibrotic and neoplastic diseases, it was hypothesized that TNF-α may be involved in the pathogenesis of keloids. Therefore, the aim of the present study was to explore the effect and mechanism of action of TNF-α in keloid formation. First, the expression of TNF-α and its main receptors, sTNFR1 and sTNFR2, was detected in the peripheral blood of patients with keloids and healthy participants, whereas keloid and normal skin tissues and fibroblast culture supernatants were cultured *in vitro*. Subsequently, keloid and normal skin fibroblasts cultured *in vitro* were stimulated using recombinant human TNF-α protein, and the effects of different TNF-α concentrations on the cell survival of the two types of fibroblasts and the underlying mechanisms were investigated, in the hope of elucidating the role of TNF-α in keloid pathogenesis and providing theoretical support and an experimental basis for the diagnosis and treatment of keloids.

Materials and methods

Surgical specimens and peripheral blood samples. Patients with keloids (n=20) and healthy control patients (n=18) who underwent surgical treatment between February 2016 and March 2018 at the Department of Plastic and Burn Surgery of West China Hospital, Sichuan University (Chengdu, China) were enrolled in the present study. All keloids included in the study shared certain characteristics and were confirmed by pathological examination. A brief description is as follows (12): Exhibits continuous growth, generally exceeding 2 years, and does not subside spontaneously. It appears as a hard, mildly tender, raised tumor with a shiny surface and, occasionally, with telangiectasia. The surface epithelium becomes thinner, ranging in color from pink to purple, and may be accompanied by hyperpigmentation. The boundary is clearly delineated, but the outline is irregular. The patient may experience itching and pain in the affected area.

The inclusion criteria patients with keloids were as follows: i) Keloid diagnosed by a plastic surgeon; ii) no keloid treatments, such as radiotherapy and injection therapy, during the previous 5 years; and iii) no ulceration or infection in keloid tissue or surrounding skin.

The exclusion criteria patients with keloids were as follows: Patients i) diagnosed with malignant tumors; ii) diagnosed with psychological diseases; iii) diagnosed with metabolic diseases; iv) diagnosed with rheumatic immune diseases; and v) receiving radiotherapy, injection therapy and other keloid treatments over the previous 5 years.

The inclusion criteria of healthy control patients were as follows: i) Non-keloid as diagnosed by a plastic surgeon; ii) the skin had not undergone radiation or chemotherapy; and iii) no skin disease.

Table I. Demographics of participants included in the present study.

Characteristics	Keloid group (n=20)	Control group (n=18)
Age, years	28.00±6.58	31.47±10.06
Sex, male/female	9/11	6/12

Data are presented as the mean ± SD or count.

The exclusion criteria of healthy control patients were as follows: i) Exclusion criteria for patients with keloids as aforementioned; patients ii) diagnosed with inflammatory or immune responses; and iii) diagnosed with a skin disorder or skin abnormality.

The demographics of the patients are summarized in Table I. There was no statistically significant difference in age and sex distribution between the two groups.

The protocol of the present study was reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University (approval no. 2014-65) on April 29, 2014 and written consent was obtained from all the participants.

Keloid samples (n=20) were obtained from the chest, shoulders or abdomen. The areas were marked with a surgical pen and collected using a scalpel following local anesthesia with 2% lidocaine. Normal skin samples (n=18) were obtained from the abdomen or chest via an incision of a 2-4-mm rim of normal skin peripheral border without lesions. A tension-free primary closure was ensured. Following excision and removal of the epidermis, connective tissue and subcutaneous fat, each sample was divided into three parts: One part was immediately stored in liquid nitrogen (-196˚C) for RNA extraction, one was immersed in 4% formaldehyde at room temperature for 24 h to be used for immunohistochemistry, and the remaining sample was used for primary culture of fibroblasts.

Fasting peripheral blood samples (1 ml) were collected from 10 patients with keloids and 9 healthy individuals, who were randomly selected from the participants used for skin sample collection, using heparin as an anticoagulant. Within 30 min of collection, the samples were centrifuged at 3,000 x g for 15 min at room temperature, and the supernatants were preserved at -80˚C.

Antibodies and reagents. Human TNF-α recombinant protein (cat. no. 300-01A) was purchased from PeproTech, Inc.; infliximab (Remicade) was from Janssen Biotech, Inc.; Johnson & Johnson; the Cell Counting Kit-8 (CCK-8; cat. no. CK04) was from Dojindo Molecular Technologies, Inc.; human TNF-α (cat. no. ZC-35733), sTNFR1 (cat. no. ZC-54321) and sTNFR2 (cat. no. ZC-54322) ELISA kits were purchased from ZCI Bio, China (http://www.zcibio.com/chanpinzhongxin_2073.html); the cDNA reverse transcription kit (PrimeScript™ RT Reagent Kit; cat. no. RR037A) was obtained from Takara Biotechnology Co., Ltd. and 2X SYBR® Green premix was from Bio-Rad Laboratories, Inc.; propidium iodide (cat. no. ST511) was from Beyotime Institute of Biotechnology; rabbit monoclonal antibodies against TNF-α (cat. no. ab6671), TNFR1

(cat. no. ab19139) and TNFR2 (cat. no. ab109322) were all purchased from Abcam; rabbit polyclonal antibodies against inhibitor of NF-κB (IκB-α; cat. no. 9242s), phosphorylated (p)-SAPK/JNK (cat. no. 4668s), p38 MAPK (cat. no. 9212), p-p38 MAPK (cat. no. 4511s), p-IκB-α (cat. no. 9246s) and SAPK/JNK (cat. no. 9252s) were all obtained from Cell Signaling Technology, Inc.; mouse polyclonal antibody against β-actin (cat. no. 60008-1-lg) was purchased from PeproTech, Inc.; horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H+L) (cat. no. ZB-2305) and HRP-labeled goat anti-rabbit IgG (H+L) (cat. no. ZB-2301) were obtained from OriGene Technologies, Inc.

Primary culture of fibroblasts. On a clean bench, tissue samples were washed with PBS solution containing 100 U/ml penicillin/streptomycin three times, cut into small pieces (0.5-1 mm^3) with eye scissors, and then seeded every 1 cm into a T25 culture bottle (Thermo Fisher Scientific, Inc.). The culture bottle was placed upside down for 2 h at 37˚C, 5% CO_2 and 95% saturated humidity, after which time the cells were supplemented with DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin. Culture was continued in the incubator until the fibroblasts grew out of the tissue block and covered the bottom of the culture bottle. Cells used for experiments were between passages 3 and 5. Keloid and normal skin fibroblasts were respectively treated with 20, 40, 80 ng/ml TNF-α for 0, 24, 48, 72 and 96 h to detect the cell survival rate using CCK-8 assay. Keloid fibroblasts were treated with 40 ng/ml TNF-α, 20 μg/ml infliximab or 20 μg/ml infliximab + 40 ng/ml TNF-α for 0, 24 and 48 h to detect the cell survival rate using CCK-8 assay and for 48 h to analyze the cell cycle by flow cytometry, and were also treated with 40 ng/ml TNF-α for 3 h to detect the phosphorylation of JNK, p38 and IκBα by western blot.

ELISA. A total of 50 μl diluted sample (100X diluted serum, or 10X diluted cell culture supernatant) were added into each well and incubated for 1 h at 37˚C, followed by the addition of 100 μl biotin antibody to each well and further incubation for 1 h at 37˚C. Each well was aspirated and washed, and the process was repeated twice for a total of three washes. Subsequently, 100 μl HRP-avidin was added into each well and incubated for 1 h at 37˚C. After washing, 100 μl 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well, followed by an incubation for 30 min at 37˚C. Stop solution (50 μl) was then added, and the optical density of each well was determined within 15 min, using a microplate reader (Multiskan™ FC; Thermo Fisher Scientific, Inc.) set to 450 nm.

Total RNA extraction and quantitative PCR (qPCR) analysis. The keloid and normal skin dermis stored in liquid nitrogen were ground in a mortar filled with liquid nitrogen and then total RNA was extracted according to the manufacturer's protocol of the RNA extraction kit (Aidlab Biotechnologies Co., Ltd.). Total RNA was quantified using a microspectrophotometer (NanoDrop 2000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.), and reverse transcribed into cDNA using the aforementioned reagent (1 h at 42˚C and 10 min at 70˚C). The qPCR primers were synthesized by Sangon Biotech Co., Ltd., and their sequences are as follows: β-actin forward, 5'-CGAGGCCCAGAGCAAGAGAG-3' and reverse, 5'-CGGTTGGCCTTAGGGTTCAG-3'; TNF-α forward, 5'-GGCAGTCAGATCATCTTCTCGA-3' and reverse, 5'-CGGTTCAGCCACTGGAGCT-3'; TNFR1 forward, 5'-CAAGTGCCACAAAGGAACCTAC-3' and reverse, 5'-CAGCTGAGGCAGTGTCTGA-3'; TNFR2 forward, 5'-CTATGACCAGACAGCTCAGATG-3' and reverse, 5'-CAGTTCCAGAGCTGGGTGTAT-3'; IL-6 forward, 5'-ATGCAATAACCACCCCTGAC-3' and reverse, 5'-CTGCGCAGAATGAGATGAGT-3'. The SYBR-Green qPCR amplification conditions were as follows: Initial denaturation for 1 min at 95˚C; 40 cycles of 10 sec at 95˚C and 30 sec at 58˚C. Sequence detection software, version 1.2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to analyze the Cq value of each amplification reaction in the qPCR, and the $2^{-\Delta\Delta Cq}$ method was used for relative quantitative analysis (13).

Immunohistochemistry. Fixed tissue was embedded in paraffin and cut into 4-μm serial sections. After dewaxing sections to water, the sections were treated with 3% hydrogen peroxide in methanol for 30 min, then washed with 0.01 M PBS for 5 min and the process was repeated three times. After blocking with 5% bovine serum albumin (Biosharp Life Sciences) in PBS for 20 min at 37˚C, the sections were incubated with diluted primary antibodies against TNF-α (1:100), TNFR1 (1:50) or TNFR2 (1:50) overnight at 4˚C, then washed with 0.01M PBS for 5 min and the process was repeated three times. Next, the sections were incubated with secondary antibodies (1:200; cat. no. ZB-2301; OriGene Technologies, Inc.) labelled with HRP for 1 h at 37˚C. After staining with 3,3'-diaminobenzidine for 10 min and hematoxylin solution for 20 sec at room temperature, the expression and distribution of the target proteins were observed under an inverted phase contrast microscope (magnification, x400; Olympus Corporation). The results were analyzed using Image-Pro Plus software 6.0 (Media Cybernetics, Inc.) and scored according to Crambert *et al* (14).

CCK-8 assay. Cells were seeded in a 96-well plate at a density of $5x10^3$ cells/well. When observed at exponential growth, cells were treated as aforementioned in the 'Primary culture of fibroblasts' section and incubated for 24, 48, 72 and 96 h. Next, 10 μl CCK-8 solution was added to each well and incubated for 4 h at 37˚C. Subsequently, the absorbance was measured on a microplate reader (Thermo Fisher Scientific, Inc.) at 450 nm. Cell viability was calculated as follows: Cell viability (%)=[treated (OD)-blank (OD)/control (OD)-treated (OD)] x100%.

Flow cytometry. The cells were harvested, fixed in 70% cold ethanol for 16 h overnight at 4˚C, and centrifuged at 300 x g for 5 min at 4˚C. The supernatants were discarded. Then, the cell pellet was stained with 0.4 ml propidium iodide staining solution in a bath at 37˚C for 30 min in the dark. Cell cycle distribution was analyzed using FACSCalibur flow cytometer (BD Biosciences), and the percentage of cells in each cell cycle phase was evaluated (FACSCalibur software version 1.3; BD Biosciences).

Western blot analysis. The cells were collected and lysed in RIPA buffer (Beyotime Institute of Biotechnology), then total

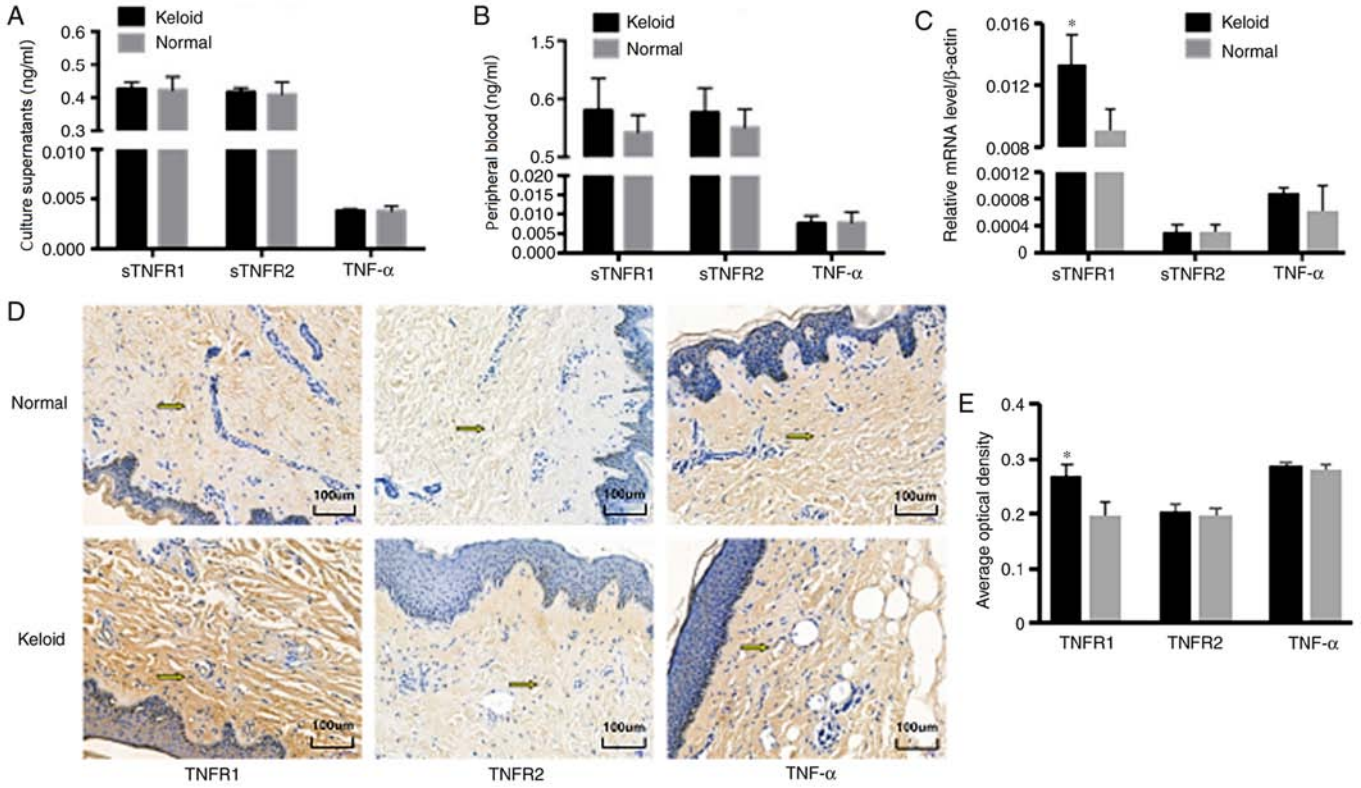

Figure 1. Expression of TNF-α, sTNFR1 and sTNFR2 in keloid tissue and normal skin. The content of TNF-α, sTNFR1 and sTNFR2 was detected by ELISA (A) in the culture supernatants of (n=9) of primary fibroblasts isolated from keloid or normal skin of patients, and (B) in peripheral blood samples (n=5) from patients with keloids or healthy participants. (C) mRNA levels of TNF-α, sTNFR1 and sTNFR2 in keloid or normal fibroblasts were detected by quantitative PCR analysis and β-actin served as the reference gene (n=6). (D) Dermis from patients with keloids or healthy participants was embedded in paraffin and used to detect the protein levels of TNF-α, sTNFR1 and sTNFR2 by immunohistochemistry (magnification, x400). (E) Results were quantitated and the mean optical density was plotted as a histogram to compare the protein levels (healthy n=9; keloid n=10). *P<0.05 vs. normal. Data are presented as the mean ± SD. TNF, tumor necrosis factor; sTNFR, soluble TNF receptor.

cell proteins were harvested, and protein concentration was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 15 μg protein per lane was loaded on 10% sodium dodecyl sulfate polyacrylamide gel (Bio-Rad Laboratories, Inc.), protein electrophoresis was performed (120 V, 1 h), followed by a transfer to a PVDF membrane (90 V, 90 min), which was then blocked with 5% non-fat milk for 2 h at 4˚C. The membrane was then incubated with the aforementioned primary antibodies against IκB-α (1:1,000), p-IκB-α (1:1,000), p38 MAPK (1:1,000), p-p38 MAPK (1:1,000), SAPK/JNK (1:1,000), p-SAPK/JNK (1:1,000) and β-actin (1:5,000) overnight at 4˚C. Subsequently, the PVDF membranes were washed three times in Tris-buffered saline with 0.1% Tween-20 (TBST), and incubated with the aforementioned HRP-conjugated secondary antibodies (1:5,000) for 2 h at 37˚C. After washing with TBST and incubation with an ECL solution (Bio-Rad Laboratories, Inc.), the membranes were exposed and images were captured. Quantity One version 4.6.6 software (Bio-Rad Laboratories, Inc.) was used to analyze the expression of target proteins and internal reference protein β-actin.

Statistical analysis. The data are presented as the mean ± standard deviation and statistical analysis was performed using SPSS 16.0 (SPSS Inc.). For normally distributed data, independent samples t-test was applied to analyze differences between two groups, while one-way ANOVA followed by Tukey's post hoc was used to determine the statistical significance of the differences among multiple groups, and Welch's test followed by Games-Howell post hoc test was applied to data with unequal variances. Non-parametric Mann-Whitney test was applied to data with non-normal distribution. P<0.05 was considered to indicate statistically significant differences.

Results

Expression of TNF-α, sTNFR1 and sTNFR2 in keloid and normal skin. Fibroblasts from keloid and normal skin were isolated, and the content of TNF-α and its receptors, sTNFR1 and sTNFR2, was detected in the fibroblast culture supernatants by ELISA. There was no significant difference in the content of TNF-α, sTNFR1 or sTNFR2 in the culture supernatants between keloid and normal skin fibroblasts (P>0.05; Fig. 1A). To further verify these results, peripheral blood samples were obtained from patients with keloids and healthy participants, and the content of TNF-α, sTNFR1 and sTNFR2 in the plasma was detected by ELISA. As in culture supernatants, there was no statistical difference in the content of TNF-α, sTNFR1 or sTNFR2 in the plasma between keloid and normal skin (P>0.05; Fig. 1B). Surgical specimens of keloids and normal skin were used to study the expression of the aforementioned molecules at the mRNA and protein

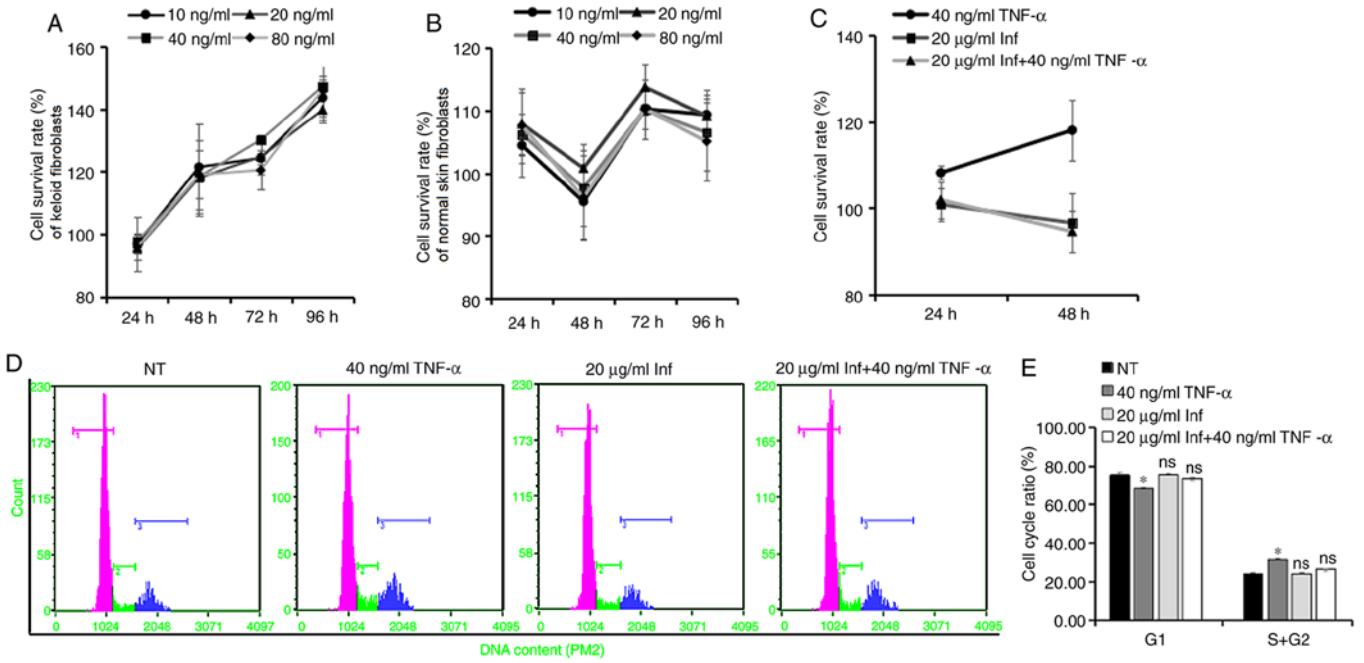

Figure 2. TNF-α regulates the proliferation of keloid fibroblasts. (A) Keloid or (B) normal skin fibroblasts were stimulated with different concentrations (0, 10, 20, 40 and 80 ng/ml) of TNF-α recombinant protein, and the CCK-8 assay results were used to evaluate the effect of the treatment on cell proliferation (n=6). The untreated group at each time point was set to 100% and compared with the cell survival rates of the corresponding treated groups. Keloid fibroblasts were pretreated with Inf, and were subsequently stimulated with or without TNF-α recombinant protein (40 ng/ml). (C) CCK-8 assay results were used to evaluate the effect of the treatment on cell proliferation (n=6). (D) Cell cycle distribution was analyzed after 48 h treated with TNF-α and infliximab by flow cytometry (rose color indicates the G_1 cell count; green indicates the S phase cell count; and blue indicates the G_2 phase cell count), and (E) cell cycle ratio was plotted as a histogram to compare the number of cells in different phases of the cell cycle (n=3). *P<0.05 vs. NT. Data are presented as the mean ± SD. TNF, tumor necrosis factor; CCK-8, Cell Counting Kit-8; Inf, infliximab; ns, not significant; NT, non-treated.

level, by qPCR analysis of fibroblasts cultured *in vitro* and by immunohistochemistry and microscopic examination of tissue samples, respectively. The results demonstrated that the mRNA levels of TNF-α and sTNFR2 in fibroblasts did not differ significantly between the two groups (P>0.05), while the sTNFR1 mRNA level in KFs was significantly higher compared with that in normal skin (P<0.05; Fig. 1C). On immunohistochemical analysis, the TNF-α and sTNFR2 protein levels exhibited no significant difference between the two groups (P>0.05), while the sTNFR1 protein level in keloid tissue was significantly higher compared with that in normal skin (P<0.05; Fig. 1D and E). These data suggested that there was no difference in the expression of TNF-α and sTNFR2 in the peripheral blood, skin tissues and fibroblasts cultured *in vitro* between patients with keloids and healthy individuals; however, the expression of sTNFR1 in keloid tissue was found to be significantly higher compared with that in normal skin.

TNF-α regulates the proliferation of KFs. In order to verify this hypothesis, different concentrations (0, 10, 20, 40 and 80 ng/ml) of TNF-α recombinant protein were applied to treat KFs and normal skin fibroblasts, and the CCK-8 assay was used to evaluate the effect of the treatment on cell proliferation. The results revealed that TNF-α promoted the proliferation of KFs at 48, 72 and 96 h after stimulation (Fig. 2A), whereas it had little effect on normal skin fibroblasts (Fig. 2B). To further study the proliferation-promoting effect of TNF-α on KFs, cells were pretreated with infliximab, a specific antagonist of TNF-α. The CCK-8 assay results revealed that the proliferation-promoting effect of TNF-α on KFs was reversed by this treatment (Fig. 2C). The cell cycle analysis by flow cytometry supported the results mentioned above. Treatment with TNF-α increased the proportion of KFs in the S and G_2 phases of the cell cycle, and reduced the proportion of G_1 phase cells compared with the NT group, while pretreatment with infliximab eliminated this increased proportion of S and G_2 phase cells (Fig. 2D and E). These results indicated that TNF-α may promote the proliferation of KFs.

TNF-α regulates the NF-κB, JNK and p38 MAPK pathways in KFs. The aforementioned results demonstrated that TNF-α promotes KF proliferation. Subsequently, the expression of IL-6, which is an important effector of the NF-κB, JNK and p38 MAPK signaling pathways (11), was first detected. The IL-6 mRNA level in KFs significantly increased after TNF-α treatment (P<0.05; Fig. 3A), which suggested that TNF-α stimulation may lead to the activation of one or more of those pathways. To identify the exact pathway activated, differences in the activation levels of the NF-κB, JNK and p38 MAPK pathways were detected in KFs treated with or without TNF-α. The western blotting results revealed that, following KF stimulation with TNF-α for 30 min, the relative level of the p-JNK protein increased significantly compared with that in the untreated group (P<0.05; Fig. 3B), which indicated activation of the JNK pathway. The IκB-α protein level decreased significantly (P<0.01), accompanied by a significant increase in p-IκB-α (P<0.05; Fig. 3C), which indicated activation of the NF-κB pathway. The relative level of the p-p38 protein also increased significantly after treatment with TNF-α (P<0.05; Fig. 3D), which indicated activation of the p38 MAPK pathway.

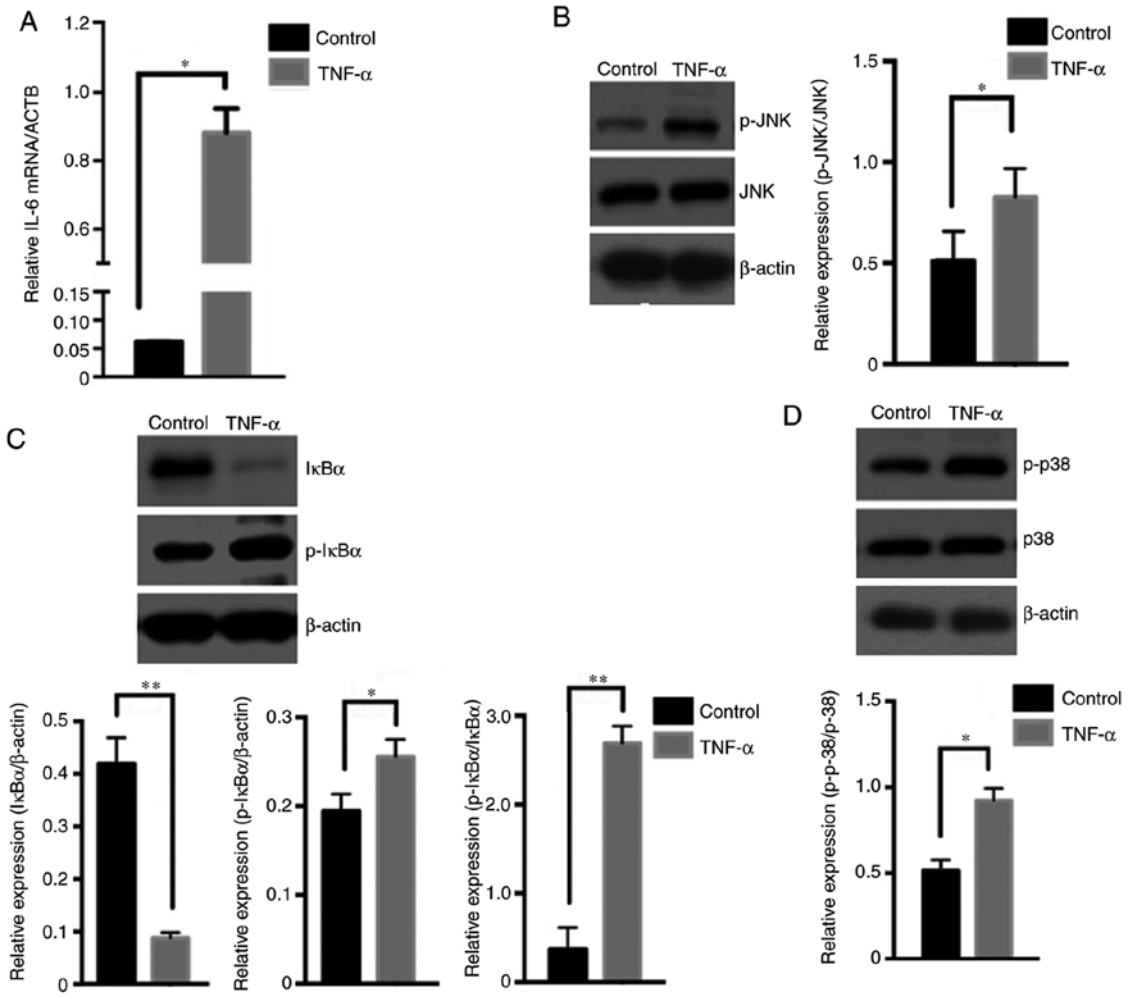

Figure 3. TNF-α regulates NF-κB, JNK and p38 MAPK pathway activation in keloid fibroblasts. (A) Keloid fibroblasts were stimulated with or without 40 ng/ml TNF-α recombinant protein for 24 h, and the IL-6 mRNA level in keloid fibroblasts was detected by quantitative PCR analysis, with ACTB serving as the reference gene (n=6). Keloid fibroblasts were stimulated with or without 40 ng/ml TNF-α recombinant protein for 30 min, and the protein levels of (B) p-JNK, JNK and β-actin, (C) IκB-α, p-IκB-α and β-actin, and (D) p-p38, p38 and β-actin were detected by western blotting (n=3). *P<0.05 and **P<0.01 vs. control. Data are presented as the mean ± SD. TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; MAPK, p38 mitogen-activated protein kinase; IL, interleukin; IκB-α, inhibitor of NF-κB; ACTB, β-actin; p, phosphorylated.

These data confirmed that TNF-α simultaneously activated the NF-κB, JNK and p38 MAPK pathways in KFs.

Discussion

The present study revealed that TNF-α may play an important role in the hyperproliferation of KFs. A previous gene chip study confirmed that the expression of pro-inflammatory factors, such as IL-1α, IL-1β, TNF-α and IL-6, was upregulated in KFs. McCauley *et al* (8) found that the TNF-α protein level in the serum of African American patients with keloids was increased compared with that in normal subjects without keloids, and Messadi *et al* (9) observed that the TNF-α mRNA and protein levels in keloid tissue were higher compared with those in surrounding normal skin using specific cDNA microarrays, western blot analysis and immunohistochemistry. However, in the present study, the expression of TNF-α in the plasma, tissue specimens and culture supernatants of skin fibroblasts did not differ significantly between patients with keloids and healthy individuals, which is inconsistent with the results of previous studies. It may be hypothesized that the inconsistency for plasma test results may be due to the differences among different ethnicities and geographic regions, since McCauley *et al* (8) collected blood samples from African American patients, while the samples collected in the present study were collected from Asian patients. Furthermore, McCauley *et al* (8) detected the change in TNF-α level in peripheral blood mononuclear cells, which is different from the direct detection of serum TNF-α level in the present study. The inconsistency for tissue test results may be associated with the processing of surgical specimens. In the present study, the epidermis and connective tissue were removed from the skin samples, and only the dermis was retained. Thus, the expression level of TNF-α was only detected in fibroblasts, which helped to more accurately study its function in these cells.

There are two main receptors of TNF-α: TNFR1 (also referred to as p55 or CD120a) and TNFR2 (also referred to as p75 or CD120b). Both receptors are classed as type I transmembrane proteins. TNFR1 is expressed in almost all tissues and cells, and may be activated by two forms (soluble and membrane bound) of TNF-α; TNFR2 is mainly expressed in immune and endothelial cells, and mainly interacts with

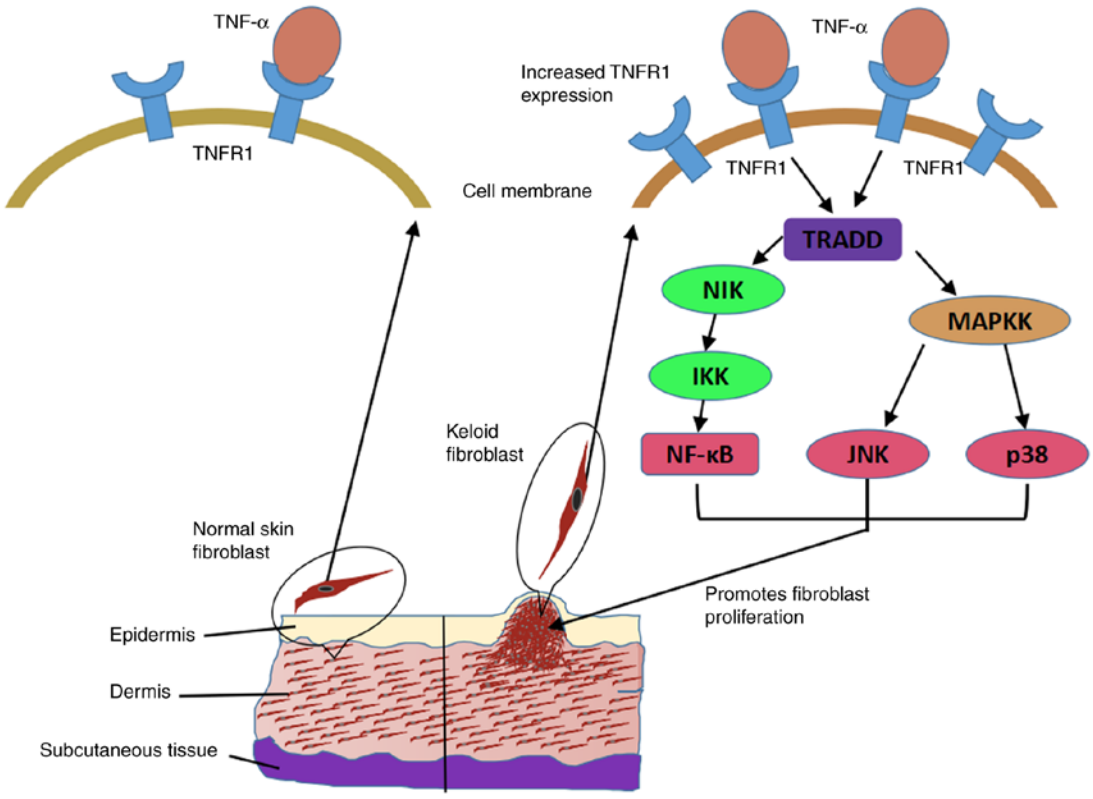

Figure 4. TNF-α promotes KF hyperproliferation by activating the NF-κB, JNK and p38 MAPK pathways. Increased expression of TNFR1 in KFs compared with that in normal fibroblasts may contribute to their enhanced sensitivity to TNF-α, and then the persistent stimulation by a low concentration of TNF-α may lead to repeated activation of the NF-κB, JNK and p38 pathways, thereby promoting the sustained and excessive proliferation of KFs. TNF, tumor necrosis factor; TNFR, TNF receptor; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; MAPK, p38 mitogen-activated protein kinase; KF, keloid fibroblast; NIK, NF-κB inducing kinase; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein.

membrane bound TNF-α. TNFR1 mediates almost all known biological effects of TNF-α. The activation of TNFR1 can further activate a variety of signal transduction pathways by recruiting a series of intracellular adaptor proteins (7,11). Since sTNFR1, the main receptor of TNF-α, has been found to be more highly expressed in keloid tissue compared with normal skin, it was hypothesized that KFs exhibit increased sensitivity to TNF-α compared that in normal skin fibroblasts. The expression of the TNF-α receptors sTNFR1 and sTNFR2, was also detected in the current study. There was no significant difference in the expression of TNFR2 between keloid and normal skin, but the expression of TNFR1 in keloid tissues and fibroblasts cultured *in vitro* was higher compared with that in normal skin. It was previously reported that the expression of TNFR1 in normal skin was significantly higher compared with that in keloid tissue (15), but this was contradictory to the hypothesis that the upregulation of TNFR1 inhibits apoptosis and promotes the proliferation of fibroblasts. In addition, Peruccio *et al* (16) observed that the TNFR1 mRNA level in hypertrophic scars after burn injury was lower compared with that in keloids. This conclusion was different from the findings of the present study, as hypertrophic scars after burn injury were not examined in the present study, and it may be hypothesized that this is due to the different scar types and testing sites of surgical specimens, as previous studies either focused on hypertrophic scars or did not explicitly mention the testing sites of specimens (15,16). Based on the results of the present study, as TNFR1 is the main receptor of TNF-α mediating its biological effects, its higher expression may lead to more potent sensitization of KFs to the stimulatory action of TNF-α.

Hyperproliferation of fibroblasts is the main cause underlying keloid formation and progression (3). Previous studies have demonstrated that TNF-α is involved in various fibrotic diseases. Miyazaki *et al* (17) found that transgenic mice expressing high levels of TNF-α in the lung were more susceptible to fibroalveolitis compared with those with low levels of TNF-α. Guo *et al* (18) reported that the renal interstitial volume of $TNFR1^{-/-}/TNFR2^{-/-}$ double knockout mice was significantly lower compared with that of the control group. Gurevitch *et al* (19) found that cardiac fibroblasts were able to proliferate and transform into myofibroblasts under TNF-α stimulation. Weiner *et al* (20) demonstrated that TNF-α directly promoted the proliferation of Ito cells and fibroblasts in the liver, and Elias *et al* (21) reported that TNF-α promoted the proliferation of lung fibroblasts. All these previous findings indicate that TNF-α can promote fibroblast proliferation. In the present study, the regulatory effect of TNF-α on the proliferation of KFs was investigated. The results demonstrated that 40 ng/ml TNF-α could promote the proliferation of KFs, but not that of normal fibroblasts. As an increased expression of TNFR1 potentially increases the sensitivity of KFs to TNF-α, it may be hypothesized that, during the process of keloid formation, KFs with a higher sensitivity to TNF-α are under constant stimulation. Consequently, the intracellular signaling pathways are repeatedly activated, eventually leading to sustained and excessive fibroblast proliferation.

The present study further investigated the mechanism underlying the role of TNF-α in the regulation of KF proliferation. After binding to TNFR1, TNF-α may activate NF-κB, JNK, p38 MAPK, or other intracellular signaling pathways. Since the activation of these signaling pathways may differ among different cell types, the specific pathways activated in KFs have not yet been fully elucidated. NF-κB is the most important signaling molecule for TNF-α to exert its promoting effect on cell proliferation and inflammation (10,22), whereas the JNK and p38 MAPK pathways may also promote cell survival and proliferation (11,22,23). In the present study, it was observed that TNF-α simultaneously activated the NF-κB, JNK and p38 MAPK pathways in KFs, which suggested that the proliferation of KFs induced by TNF-α stimulation was closely associated with the activation of these pathways. This was consistent with previous findings reporting the promotion of cell proliferation by these pathways (7,11,22,23). Therefore, it was inferred that the persistent activation of the NF-κB, JNK and p38 pathways may be the mechanism underlying TNF-α-induced KF proliferation (Fig. 4).

In conclusion, compared with normal fibroblasts, increased expression of TNFR1 in KFs may confer a unique sensitivity to TNF-α, and persistent stimulation by a low concentration of TNF-α may lead to repeated activation of the NF-κB, JNK and p38 pathways, thereby promoting the sustained and excessive proliferation of KFs.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Sichuan Basic Applied Research Project (grant no. 2017FZ0055), the Post-Doctor Research Project, West China Hospital, Sichuan University (grant no. 2018HXBH082) and the China Postdoctoral Science Foundation (grant no. 2019M653413).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QL and FC participated in the design of the experiments, optimization of experimental conditions, conduction of the experiments and writing of the manuscript. KZ, LF and JW participated in the recruitment of participants and the collection of clinical specimens. QX and YC participated in the experimental design and data analysis. JC and YQ participated in designing the present study and revising the manuscript and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the present study was reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University written consent was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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