Topical Application of Naringenin inhibits Hypertrophic Scar formation by Suppressing Fibroblast Activation and Local Inflammation

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ABSTRACT

The pathogenesis and therapy of hypertrophic scar (HS) have not yet been established. The aim of our study is to investigate the potential effect of naringenin on HS and its underlying mechanisms. The mouse model with HS was prepared by mechanical stretch device and then treated with naringenin at various concentrations. Histological studies were performed to evaluate scar hypertrophy by haematoxylin and eosin and Masson’s trichrome staining. The activation of HS fibroblasts was determined based on real-time PCR, Western blot and immune-histochemical staining. After observing the retention of inflammation cells by immune-histochemistry, the cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and transforming growth factor-beta 1 (TGF-β1), mRNA and protein levels were quantitated by real-time PCR, ELISA and Western blot methods. Naringenin significantly inhibited the formation of HS in a concentration-dependent manner. Naringenin also inhibited fibroblasts activation and inflammatory cells recruitment. In addition, mRNA and protein expression levels of TNF-α, IL-1β, IL-6 and TGF-β1 were down-regulated after naringenin treatment. This study highlighted new pharmacological activity of naringenin on HS. The mechanism of action of naringenin was associated with the inhibition of fibroblast activation and local inflammation. These results suggested that naringenin could serve as a novel agent for treatment of HS.

Key words: Hypertrophic scar, naringenin, fibroblast, inflammation.

INTRODUCTION

Cutaneous injury triggers an immediate and cascade of repair events which can be summarized as the normal healing response which culminates in scar formation (Bran, 2009). However, aberrant wound healing process results in abnormal scar formation such as hypertrophic scar (HS), which is red, raised and sometimes itchy, resulting in functional and cosmetic defects and mental pressure (Mustoe et al, 2002; Butzelaar, 2015). Currently, there is no effective treatment for HS, and nearly 35% of surgical skin wounds give rise to HS after one year (Bran, 2009). Therefore, valid approach for HS treatment is urgently needed to be researched and developed.

The exact mechanism of HS formation remains unclear, but potential factors thought to play a dominant role in human HS formation include mechanical force (Aarabi, 2007), local inflammation (Gol, 2008; Verri et al, 2006) and fibroblast activation (Sarrazy et al, 2011). Exaggerated inflammatory phase is closely associated with HS development, in which inflammatory cells such as neutrophils, macrophages and T-lymphocytes migrate to the wound site with high secretion of various cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and transforming growth factor-beta 1 (TGF-β1) (Verri et al, 2006; Salgado et al,
Specific cytokines and inflammatory factors, such as TGF-β1, contribute to fibroblast activation and the modulation of the fibroblast phenotype (Ren et al, 2015). Many studies have reported that over-activation of dermal fibroblasts is involved in the process of HS formation (Verri et al, 2006; Zhu et al, 2013). Activated fibroblasts trans-differentiate into myofibroblasts which contribute to the accumulation of collagens that are responsible for fibrosis (Francis et al, 1989). Simultaneously, in addition to their profibrotic properties, activated fibrocytes are reported to produce pro-inflammatory mediators such as IL-6 and interleukin-8 (IL-8), which participate in the early events mediating inflammation (Peng and Herzog, 2012). Therefore, inflammation events and fibroblast activation are potential therapeutic targets for the treatment of HS. Since the mid-1960s, intra-lesional corticosteroid injection has been crucial to treating HS (Butzelaar, 2015) through decreasing the severe inflammatory state in the wound (Niessen et al, 1999). Nonetheless, side effects, for example, telangiectasia, rebound effects, skin atrophy, hypopigmentation, injection pain and ineffectiveness occur at high frequency (Arno et al, 2014). There are currently no valid treatment methods available for HS that prevent fibroblast activation. Thus, it is crucial to develop a more effective strategy to regulate this inflammatory process as well as, fibroblast activation to prevent hypertrophic scarring.

As a natural predominant flavanone, naringenin (4’,5, 7-trihydroxyflavanone) has an extensive scope of pharmacological characteristics, such as anti-tumor (Van Der Veer et al, 2009) anti-mutagenic (Fette, 2006) and anti-atherogenic (Lee et al, 2001) activity. Furthermore, naringenin has been proved to have anti-inflammatory effects in a number of illnesses. Naringenin has greatly reduced the symptom of colitis, down regulating the pro-inflammatory mediators, for instance, IL-6 and TNF-α in colon mucosa (Desmouliere et al, 2005). It has been suggested that naringenin shows anti-inflammatory effect on chronic bronchitis by reducing the concentrations of IL-8, leukotriene B4 and TNF-α in bronchoalveolar lavage fluid (Luo et al, 2012). In addition, naringenin down-regulated the release of pro-inflammatory cytokines such as TNF-α and IL-1β, which abrogated the ischemic brain injury through the suppression of NF-kB-mediated neuro-inflammation (Raza et al, 2013). On the other hand, it has been proven to attenuate interstitial fibrosis in pressure induced-cardiac hypertrophy through PI3K/Akt, ERK and JNK signaling pathways (Zhang et al, 2016) and to inhibit renal fibrosis by blocking Smad3 phosphorylation and transcription (Mustoe et al, 2002).

Given these evidences, we wonder what effect naringenin would have on dermal fibroblasts and whether naringenin could inhibit dermal inflammation and HS formation in vivo. Consequently, we investigated its function in the HS model induced by mechanical load. Furthermore, we investigated its effects on distinct aspects of local inflammation such as inflammatory cell infiltration and inflammatory cytokine release, and tested its effects

**MATERIALS AND METHODS**

**Animals and hypertrophic scar model**

Shanghai Slac Laboratory Animal (Slac, Shanghai, China) provided female KM mice which were eight weeks old for the experiment. School of Medicine, Shanghai Jiaotong University Animal Care and Use Committee approved the whole process and all the animals were kept in standardized conditions under guidelines approved by the institution. Based on a published approach, the model of HS was built. Briefly, a 2-cm incision was made and sewed in every mouse’s dorsal midline. After four days, the sutures were moved away. Mechanical stretch devices were sewed covering the incisions, and after the incision, stretch was conducted from day 4 to 14. The animals were grouped into three categories at random with eight mice in every group. Group A was the controlling group, taking DMSO every day. After incision, Groups B and C were the model of HS, taking naringenin (dissolved in DMSO) with a concentration of 25 μM and a concentration of 50 μM in groups B and C respectively for exterior usage of once daily from day 4 to 14. On day 14 (24 h after the last usage of drug), all the mice were killed and the specimens of scar collected for future study.

**Histology and immunohistochemistry**

The 5 μm paraffin-embedded and paraformaldehyde-fixed tissue sections were stained with hematoxylin and eosin (H and E) and Masson’s trichrome (Trichrome stain LG solution, HT10316; Sigma-Aldrich). Regarding immunohistochemical staining, the sections were found with primary antibodies against α-SMA (Abcam; 1:200), CD4 (Abcam; 1:200), CD68 (Abcam; 1:200) at 4°C overnight. After being incubated with the proper secondary antibodies, the sections were counterstained with hematoxylin and developed with diaminobenzidine. Utilizing Adobe Photoshop CS4, the images were processed for publication purpose.

**Quantitative polymerase chain reaction**

Liquid nitrogen was added, the skin of mouse was thoroughly grounded with a pestle and mortar and then evenly divided with a syringe and needle. Trizol purification (Invitrogen, Carlsbad, CA, USA) was utilized to isolate total RNA which was submitted to reverse transcription with M-MLV Reverse Transcriptase (Thermo Fisher Scientific) and Oligo (dT). Synthesized complementary DNA (cDNA) was analyzed with
quantitative real-time PCR using SYBR®Premix (Takara, Dalian, China) and the 7900 HT FastReal-Time PCR system (Life Technologies). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as a reference gene. dehydrogenase (GAPDH) was utilized as a reference gene. Primers sequences were: α-SMA, 5'-GTCCGACATCAGGAGTAA-3' (forward) and 5'-TGGATACTTGCGTCAGGA-3' (reverse); IL-1β, 5'-GAATGCCACCTTGTGAC-3' (forward) and 5'-TGATGCTCTCATAACGAGC-3' (reverse); IL-6, 5'-TCTATACCCTTCAAGTCGGA-3' (forward) and 5'-GAATGCCATGGCACAACCTT-3' (reverse); TGF-β1, 5'-CCACCTGCAAGACCATCGAC-3' (forward) and 5'-CTGGGAGCCTTAGTTGGA-3' (reverse); TNF-α, 5'-CAGCCGCTGCTATGTCCTC-3' (forward) and 5'-CGATCACCCGAAATCTAGTAG-3' (reverse).

**Western blot assay**

Skin tissues were added to radio immuno-precipitation assay (RIPA) lysis buffer and homogenized. After absolute schizolysis, the homogenate was centrifuged at 4°C for 10 min at 12,000 rpm. Also, the supernatant was gathered as the sample of protein. The bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) was used to determine the concentrations of protein. The proteins at the dose of 20 μg were isolated by 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted in the membranes of polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA, USA). Later, the specific protein binding sites on the membranes were blocked with 0.1% of Tween-20 (TBST) and 5% of dried nonfat milk in Tris-buffered saline (0.5M NaCl and 20 mMTris, pH 7.6) at room temperature for 1 h. The membranes were gently shaken and incubated with major antibodies against the antibodies of TNF-α (Abcam; 1: 500), TGF-β1 (Abcam; 1: 500), IL-6 (Abcam; 1: 500), IL-1β (Abcam; 1: 500), GAPDH (Bioworld; 1: 10000), and α-SMA (Abcam; 1: 500) at 4°C overnight. In the following day, the membranes were incubated with peroxidase-conjugated secondary antibody (1:1000) (Nebraska, USA) at room temperature for 1 h after washing with TBST. Using Image J software, quantitative analysis was conducted on immuno-reactive bands.

**Sandwich ELISA method**

Based on the instruction of manufacturer, the expression degrees of IL-1β, IL-6, TNF-α and TGF-β1 in the extract of mouse skin scar tissue (n=8, each group) were found by the approach of sandwich ELISA utilizing a ELISA kit from the R and D system (Minneapolis, MN, USA). According to the color reaction of antibodies and cytoplasm extract, the values of absorbance can be identified at 450 nm on a Microplate Reader.

**Statistical analysis**

All values were expressed as means ± standard deviation (SD). Analysis of the data was conducted utilizing post-hoc least significant difference (LSD) test and Friedman’s analysis of variance (ANOVA). The difference between groups was regarded considerable at P<0.05.

**RESULTS**

**Naringenin attenuated scar formation**

We first investigated whether topical application of naringenin have anti-fibrotic effects on the HS mouse model during scar formation, mice were treated with naringenin at the dose of 25 and 50 μM respectively. After exterior use of naringenin for 10 days (from day 4 to 14 after incision), in comparison to the vehicle group of DMSO-treated mice, the groups with the treatment of naringenin had shown greatly decreased formation of scar with dose-dependency. As shown in Figure 1a, the gross scar section area decreased in varying degrees after the treatment of naringenin. Alternatively, H and E staining and masson’s trichrome staining showed that cross section of the scar and scar elevation index decreased, respectively (Figure 1b and c). These results indicated that topical application of naringenin on HS mouse model had anti-fibrotic effects and inhibited scar formation induced by mechanical stretch.

**Naringenin inhibits fibroblast activation**

The degrees of α-SMA expression in the scar section of HS mouse model were evaluated in order to investigate the effect of naringenin on fibroblast activation. Compared with vehicle group, α-SMA expression was considerably decreased in the fibrotic tissues of naringenin-treated mice with dose dependency. According to the study, the mRNA expression and protein production of α-SMA in the model of HS mouse were greatly reduced by the treatment of naringenin (Figure 2a and b). Furthermore, the cells were immune-stained for the presence of α-SMA. Also, the rate of α-SMA positive cells was much higher in vehicle mouse sections than that in the scar sections of naringenin-treated mice (Figure 2c). These findings demonstrated that the activation of fibroblasts induced by mechanical stretch and injury on the model of HS mouse could be inhibited by naringenin administration.

**Naringenin suppresses inflammatory cell infiltration**

To study the effects of naringenin on inflammatory cell, cells were immuno-stained for the presence of CD68+ and
Figure 1. Decreased scar formation in naringenin-treated mouse. Inhibition effects of naringenin (Nar) on scar hypertrophy by images of gross scars sections (A), haematoxylin and eosin (H&E) stained sections (B), and Masson’s trichrome stained sections (C). Scar elevation index (SEI) were measured. ‘D’ and ‘d’ serve as the thickness of the scar and bordering normal skin, respectively. SEI is defined as the D/d ratio. The dotted lines draft the scar. Data are expressed as Mean ± SD of eight mice per group. *P < 0.05; **P < 0.01; ***P < 0.001. DMSO, dimethyl sulfoxide control.

Figure 2. Decreased activation of fibroblast in naringenin-treated mouse scar tissues. Inhibition effects of naringenin (Nar) on α-SMA by real-time PCR (A), Western blot (B) and Immunohistochemical staining (C). Data are expressed as Mean ± SD of eight mice per group. *P < 0.05; **P < 0.01; ***P < 0.001. DMSO, dimethyl sulfoxide control.
Figure 3. Decreased infiltration of CD68+ and CD4+ cells in naringenin-treated mouse scar tissues. Inhibition effects of naringenin(Nar) on CD68+ (A) and CD4+ (B) cells infiltration by Immunohistochemical staining. Data are expressed as Mean ± SD of eight mice per group. *P < 0.05; **P < 0.01; ***P < 0.001. DMSO, dimethyl sulfoxide control.

CD4+ cells. Immunohistochemistry data indicated that, in comparison to vehicle group, the CD68+ and CD4+ cells in the scar tissues of a naringenin-treated mouse were greatly decreased with dose dependency (Figure 3a and b). This result indicated that the inflammatory response could be suppressed by naringenin through decreasing the infiltration of inflammatory cells on the model of HS mouse.

Naringenin reduces the expression of inflammatory cytokine

To investigate the effects of naringenin on inflammatory cytokine, IL-1β, IL-6, TGF-β1 and TNF-α genes expression were observed by quantitative polymerase chain reaction. As a result, IL-1β, IL-6, TGF-β1 and TNF-α in naringenin-treated mouse scar tissues were significantly reduced at the concentration dose of 25 and 50 μM respectively as compared with vehicle group (Figure 4a). In addition, the protein expression level detected by ELISA and western blotting indicated that naringenin significantly down-regulated IL-1β, IL-6, TGF-β1 and TNF-α expression in a dose-dependent manner in HS mouse model, respectively (Figure 4b and c). Collectively, these data suggested that naringenin treatment on HS mouse model reduced infiltration of inflammatory cell and production of inflammatory cytokine.

DISSCUSSION

HS is a complex fibro-proliferative disorder and its definitive mechanism is still not clearly understood. Various invasive and non-invasive options are currently available for the prevention and treatment of HS. The invasive methods include corticosteroid intralesional injections, cryotherapy, laser treatment, radiation therapy and surgical excision (Meng et al, 2015). In contrast, the non-invasive methods which are easy and comfortable to use and low in expenses are becoming more and more popular. A growing body of evidences proves that the compression therapy is effective in healing scars (Ray et al, 2013; Zhang et al, 2015). However, pressure therapy also has some defects, such as the limitation in bending, anatomic compression, or fast moving regions. Furthermore, as patients have to wear the clothes all the time, they may feel uncomfortable or even suffer from skin ulcer due to uneven pressure distribution [53]. Therefore, a vital problem may lie in patients’ non-compliance which is reported to be 8.5 to 59% (Lee et al, 2001; Kealey et al, 1990).

Silicone gel sheeting has been widely used in clinics for the treatment of HS since early years (Niessen et al, 1999) and it is effective in preventing HS according to several researches (Gold, 2008; Chernoff, 2007). Nevertheless, according to a systematic review published in 2013, there was no sufficient evidence to prove the efficacy of silicone gel sheeting in preventing high-risk individuals from abnormal scarring (Peng and Herzog, 2012).

In addition, silicone has many unavoidable shortcomings, including excessive sweating, skin maceration and itching (O’Brien and Jones, 2013; Fette, 2006) and its accurate mechanism keeps blurry. Another product for treating scars is onion extract, which is believed to exhibit anti-inflammatory, bacteriostatic, and collagen down-regulatory properties (August, 1996) and improve collagen formulation in the rabbit ear models (So et al, 1997). However, according to clinical research data, onion extract
Figure 4. Decreased production of IL-1β, IL-6, TGF-β1 and TNF-α in naringenin-treated mouse scar tissues. Effects of naringenin (Nar) on the mRNA and protein expressions of IL-1β, IL-6, TGF-β1 and TNF-α were determined by real-time PCR (A), ELISA (B) and Western blot (C). Data are expressed as Mean ± SD of eight mice per group. *P < 0.05; **P < 0.01; ***P < 0.001. DMSO, dimethyl sulfoxide control.

is not able to improve hypertrophic scarring (Johnson et al, 1994; Chung et al, 2006).

Accordingly, compounds to treat HS with less side effects and optimized treatment methods should be determined. In our research, we observed that topical administration of naringenin suppressed HS development without systemic naringenin suppressed HS development without systemic health damage and the drug delivery way greatly alleviate the pain caused by traditional intralesional injection. Thus, our research estimates that local application of naringenin is a possible optimum method for treating HS.

The fibroblast is a crucial cell during scarring. However, the fibroblasts which are quiet when the skin is in a good condition will be activated once the skin is hurt (Honardoust et al, 2012). Different from the scarless healing, the constant activation of fibroblasts is kept available in abnormal scars (Jackson and Shelton, 1999).

In the process of HS formation, fibroblasts are unduly stimulated and trans-differentiated into myofibroblasts (Desmouliere et al, 2005; Hinz, 2007). Myofibroblasts express excessive α-SMA (Van Der Veer et al, 2009) which is closely related to scar contracture and texture (Honardoust et al, 2012; Wang et al, 2011) and produce massive extracellular matrix (ECM) proteins (Zurada et al, 2006). Therefore, fibroblasts have become the possible therapeutic target for anti-fibrosis treatment. Previously, naringenin has been reported to show anti-fibrosis outcomes in several fibrotic diseases such as lung fibrosis (Du et al, 2009) and liver fibrosis (Luo et al, 2012). In our studies, we investigated the effects of naringenin on fibroblasts derived from HS for the first time. Results showed that the mRNA and protein levels of α-SMA in naringenin-treated scars was lower, which thus, demonstrated that naringenin could decrease scar formation by inhibiting HS-derived fibroblasts activation.

Inflammation clearly plays a major role in HS progression.
Compared to the normal scarring, HS presents an enhanced and prolonged inflammation phase characterized by a larger number of inflammatory cells recruitment and inflammatory cytokines infiltration (Hinz et al., 2007; Verri et al., 2006; Chernoff et al., 2007). It is reported that macrophages (Yagmur et al., 2010) and lymphocytes, especially CD4+ T lymphocytes (Wynn and Barron, 2010; Wynn, 2004) are the dominant inflammatory cells in HS pathology. These inflammatory cells prompt exaggerated scar formation by modulating the fibroblast phenotype and secreting cytokines such as IL-1β, IL-6, TGF-β1 and TNF-α. Elevated levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were observed in HS (Sarrazy et al., 2011; Raza et al., 2013). Herein, we demonstrated that treatment with naringenin inhibited the CD4+ T lymphocyte and CD68+ cells (monocyte/macrophage) retention as well as, pro-inflammatory cytokines levels such as IL-1β, IL-6 and TNF-α. Moreover, naringenin inhibited TGF-β1 production in HS. In the cases of acute inflammation, TGF-β1 belongs to anti-inflammatory cytokines which are generated together with the pro-inflammatory cytokines and used to inhibit inflammatory reaction (Wong et al., 2011). In this context, the suppression of IL-1β, IL-6 and TNF-α production might result in the reduction of TGF-β1 levels.

CONCLUSIONS

In summary, our research shows that topical application of naringenin to hypertrophic scar mouse model can suppress HS formation. Further analyses suggests that naringenin has effectively inhibits trans-differentiation of fibroblasts into myofibroblasts, suppresses inflammatory cell infiltration, as well as downregulates the expression of inflammatory cytokines. Therefore, naringenin, with its inhibitory effects on fibroblast activation and local inflammation and relatively low cost and toxicity of natural agents, could be used as a safe, alternative therapeutic agent for treating HS. However, the precise underlying mechanism needs further elucidation.

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