MicroRNA-29a induces apoptosis via increasing the Bax:Bcl-2 ratio in dermal fibroblasts of patients with systemic sclerosis

Saeideh Jafarinejad-Farsangi1, Ali Farazmand1, Mahdi Mahmoudi2, Farhad Gharibdoost2, Elham Karimizadeh1, Farshid Noorbakhsh3, Habibeh Faridani2, and Ahmad Reza Jamshidi2

1Department of Cell and Molecular Biology, University of Tehran, Tehran, Iran, 2Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran, and 3Immunology Department, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Abstract

The most prominent feature of systemic sclerosis (SSc) and other diseases associated with fibrosis is the prolonged activation of fibroblasts not eliminated by apoptosis, hence characterized by accumulation of more extracellular matrix (ECM). We tend to verify if microRNA-29a (miR-29a) as an anti-fibrotic factor could induce apoptosis in SSc fibroblasts. We did not detect apoptosis in SSc fibroblasts. We found that Bcl-2 expression was upregulated in SSc fibroblasts and the ratio of Bax:Bcl-2 in these cells was significantly lower (p = 0.02) compared to normal fibroblasts. Transfection of both SSc and transforming growth factor-β (TGF-β) stimulated fibroblasts by miR-29a mimic, significantly decreased the expression of two anti-apoptotic members of the Bcl-2 family, Bcl-2 (p = 0.0005, p = 0.01) and Bcl-XL (p = 0.0001, p = 0.006), resulted in enhanced Bax:Bcl-2 ratio and induced a high rate of apoptosis. Recently, miR-29 has been introduced as an anti-fibrotic factor with potential therapeutic effect on SSc. Until now, it has not been proposed whether there is a relationship between miR-29a and apoptosis in SSc. According to our results, it seems that miR-29a is a potent inducer of apoptosis in SSc fibroblasts and an attenuator of ECM production in these cells. MiR-29a disrupted the expression profiling of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-XL) which is the central point of dynamic life-death rheostat in many apoptotic pathways. Furthermore, dermal fibroblasts from patients with SSc showed elevation in TNF-α mRNA levels, while restoration of miR-29a decreases TNF-α production in these cells. Although further molecular studies are necessary to investigate the underlying apoptotic pathways, the present findings suggest that anti-fibrotic and pro-apoptotic properties of miR-29a could provide novel benefits toward the development of fibroblast-specific anti-fibrotic therapies.

Introduction

Systemic sclerosis is an autoimmune disease accompanied by abnormal activation of dermal fibroblasts. The etiology of systemic sclerosis (SSc) is unknown, but it seems that over-activated immune system, prolonged inflammation, imbalanced cytokines and leaky blood vessels develop autoimmunity and fibrotic responses in SSc [1–3]. Dermal fibroblasts are activated in a complex network under the control of different cytokines, enzymes and growth factors. Transforming growth factor-β (TGF-β) is the main profibrotic cytokine involved in fibrosis of SSc [4]. Fibrotic features of SSc fibroblasts are similar to those of normal fibroblasts following TGF-β treatment [5]. It has been considered that SSc fibrotic features originate from the failure in termination of normal wound healing. Activated fibroblasts are transiently present during normal healing and undergo apoptosis following tissue repair to terminate the process of fibrotic cascade [1,6]. The prominent feature of SSc is that SSc fibroblasts are not eliminated through apoptosis and extra cellular matrix (ECM) leads to the development of scars and inflexible skin [7,8].

Apoptosis is defined as genetically programmed cell death also occurring in physiological processes, such as development and organogenesis. To restore tissue homeostasis, damaged and infected cells and those not needed any more are removed through apoptosis [9–12]. The Bcl-2 family, a group of 25 proteins divided into two functional pro- (e.g. Bax, Bak, Bid, Bad and Bim) and anti-apoptotic (e.g. Bcl-2, Bcl-XL, Bcl-X, Bcl-Xs and BAG) subfamilies [13], regulates apoptosis by controlling permeabilization of mitochondrial outer membrane (MOMP) [14]. Bcl-2 is one of the important anti-apoptotic proteins that confer cell survival in different types of cells, including fibroblasts and tumor cells [15–17].
Bcl-2 and Bcl-XL dimerize with pro-apoptotic proteins, such as Bax, to suppress apoptosis [18,19]. Since many cell types trigger Bcl-2 and Bax expression to suppress or facilitate apoptosis, the ratio of Bax:Bcl-2 has been considered as the cell-autonomous rheostat to determine apoptotic threshold [19–21]. In the presence of a mitochondrial apoptotic stimulus, pro-apoptotic proteins dissociate from anti-apoptotic ones [20], recruit to the MOMP to oligomerize [22] and form the apoptotic pores [23] resulting in cytochrome c release into the cytoplasm and apoptotic cascade initiation.

MicroRNAs (miRNAs) are endogenous small RNAs (~22 nucleotides) that regulate >50% of human genes through binding to the respective mRNAs and inhibiting their translation [24]. Many genes involved in physiological and pathological processes, such as apoptosis, wound healing and skin fibrosis, are regulated by miRNAs [25–27]. MiR-29 family members (miR-29a, miR-29b and miR-29c) are well-known regulators of collagen and some of the ECM components [28,29]. Expression of MiR-29s is negatively controlled by pro-fibrotic signaling, especially via TGF-β pathway [30,31]. Currently, miR-29a are considered as anti-fibrotic miRNAs and their potential therapeutic strategies for fibrotic diseases are under investigation in animal models [28,31,32]. New findings in cancer research indicated that miR-29a also has pro-apoptotic effect through regulating Bcl-2 family proteins [33]. It has been shown that the anti-apoptotic Bcl-2 protein is a direct target for miR-29a and its expression is inhibited by this counter-regulation and apoptosis induction follows [34].

SSc is a multifactorial disease with no definite treatment. Overcoming apoptosis resistance of SSc fibroblasts could provide important insights into the development of therapeutic agents for SSc. In this study, for the first time, the involvement of miR-29a, as an apoptotic factor, in apoptosis of SSc fibroblasts was investigated. Furthermore, the possible apoptotic effect of miR-29a by evaluating the relative expression of Bax, Bcl-2 and Bcl-XL was explored.

Materials and methods

Patients

Patients with SSc attending Outpatient Rheumatology Clinic of Rheumatology Research Center (RRC), Tehran University of Medical Sciences (TUMS) and Iran Rheumatology Center, were considered for the study. Ten patients (2 males and 8 females) with diffuse SSc (dSSc) were evaluated with structured questionnaires and experienced Rheumatologists. All the patients fulfilled the modified American College of Rheumatology (ACR) criteria for SSc. Healthy controls (2 males and 8 females), with no history of skin diseases and glucocorticoid medication, were matched for age and sex with patients. All the patients and healthy controls were willing to enter the study and filled the informed consent. Human participation in this study was approved by the local ethical committee of Tehran University of Medical Sciences and Helsinki Declaration (October 2008).

Biopsy specimens and cell culture

Punch biopsies (4 mm) were obtained from the affected forearm (tight skin) of patients and normal skin of healthy controls. Dermal fibroblasts were isolated from biopsy specimens by enzymatic digestion. Briefly, biopsies were washed with phosphate-buffered saline (PBS) and then incubated in presence of dispase II (overnight) to separate epidermis from dermis. Then, incubation in collagenase Type II for ~3 h at 37°C provided single-cell suspension of dermal fibroblasts. Fibroblasts were cultured in Dulbecco-modified Eagle’s minimal essential medium with 10% fetal bovine serum and 2% penicillin streptomycin (pen-strep) at 37°C in 5% CO2 cell culture incubator. Cells taken from passages 3–5 were used for experiments.

Transfection experiments

MiR-29a mimic, inhibitor and scrambled RNA were purchased from Qiagen (Valencia, CA). miR-29a mimic, inhibitor and scrambled RNA are synthetic oligonucleotides with sense sequence to mature miRNA (mimic), or antisense harbor sequence to inhibit mature miRNA (inhibitor) or have neutral sequence just to be used as negative control (scrambled RNA). Hiperfect transfection reagent (Qiagen) was used to transfect cells with recommended doses of miR-29a mimic (10 nM), inhibitor (50 nM) and scrambled RNA (10 nM). Cell seeding and forward transfection were performed on the same day according to the manufacturer’s protocol. Cells were harvested 24 h following transfection. miRNA extraction and cDNA synthesis performed using miRNesy Mini Kit and miScript II RT Kit, respectively (Qiagen). Transfection efficiency was controlled by real-time PCR. MiR-29a and RNU6 (internal control) primers purchased from Qiagen (miScript primer assay) and their relative expression were measured using miScript SYBR Green PCR Kit (Qiagen). MiR-29a expression was upregulated by 2-fold following transfection of miR-29a mimic (Supplementary Material 1). Normal fibroblasts were transfected using TGF-β (10 ng/ml) for 48 h to establish fibrotic phenotype in vitro (Supplementary Material 2).

RNA extraction and real-time PCR

Total RNA was extracted from cells using high pure RNA isolation kit (Roche, Nutley, NJ). ReverTaid first strand cDNA synthesis kit (Thermo scientific, Wilmington, DE) was used for complementary DNA (cDNA) synthesis. Concentration of cDNA was quantified using NanoDrop 2000c spectrophotometer (Thermo scientific). Quantitative expression of Bax and Bcl-2 genes was performed with TaqMq Real-time PCR assay. GAPDH was used as the internal control. All the real-time PCR reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster, CA).

Western-blot analysis

Cultured cells were lysed with RIPA buffer to isolate total cellular proteins. Quantification of protein concentration was carried out by Bradford method. Proteins were denatured in Laemmli sample buffer and loaded in equal amounts (60 μg) on 12% SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels. After electrophoresis, protein bands transferred onto a PVDF (polyvinylidene fluoride) membrane. The transferred membrane blocked in 4% skin milk TBST (Tris-buffered saline with Tween) for 3 h and incubated
 overnight at 4 °C or 3 h at room temperature in primary antibodies from Abcam: anti-Bcl2 antibody (Ab59348), anti-Bcl-XL (Ab32310), anti-Bax antibody (Ab53154) and anti-beta actin antibody (Ab25894) as internal control. After incubation in secondary antibody for 90 min (sheep anti-rabbit Ig peroxidase conjugated from Avidenza Research Institute, Iran, Tehran), the blotted proteins were visualized with ECL (Amersham ECL Prime Western Blotting Detection Reagent).

Apoptosis detection by flow cytometry

Detection and quantification of apoptotic cells was performed using annexin-V-FLUOS kit (Roche). About 15 × 10⁴ cells per well were cultured in 6-well plates and were harvested 24 h after miR-29a mimic (10 nM) or scrambled RNA (10 nM) and 48 h after TGF-β (10 ng/ml) treatments. According to the manufacturer’s instruction, cell suspensions were washed with cold PBS and centrifuged at 200 × g for 5 min. Then incubated for 15 min in 100 µl of diluted annexin-V-fluorescein and propidium iodide (PI) buffer in dark. For all the experiments, 10000 cells were recorded per sample on a CyFlow® ML flow cytometer (Partec, GmbH, Munster, Germany) and FASC data were analyzed using PartecFloMax software (Munster, Germany).

Hoechst 33258 staining

Apoptotic cells were morphologically analyzed with Hoechst 33258 (a DNA-specific dye; sigma) staining. Cells were cultured with 4 × 10⁴ count in 24-well plates. After treatments, culture media was removed and cells fixed in cold methanol for 30 min. Then cells were labeled with 1 mg/ml Hoechst 33258 solutions for 30 min in the dark. Finally, morphological changes in chromatin were visualized by fluorescence microscope. Apoptotic rate % (AR%) calculated by dividing the number of positively stained cells to the total number of cells × 100%. For all the experiments, 300 cells were counted for each sample.

Statistical analysis

IBM SPSS version 22 software (Armonk, NY) was used for statistical analysis. Normality of variants was analyzed by Kolmogorov–Smirnov test. Two-tailed Student’s t-test was used for statistical evaluation and p values < 0.05 were considered statistically significance. Quantitative analysis of immunoblot was performed using Image J 1.4.3.67 software (NIH Image, Bethesda, MD). All data are presented as mean ± SEM.

Results

Relative expression of Bcl-2 and Bax in SSc fibroblasts

Using real-time PCR and Western blotting, the expression level of two anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) genes were first determined in SSc fibroblasts (Figure 1). Relative expression of mRNAs was estimated from real-time PCR Cq values with 2^(-ΔΔCq) method. Our results revealed an increased expression level of Bcl-2 mRNA (1.6 ± 0.63-fold) in SSc fibroblasts, but the expression of Bax mRNA was not significant (p = 0.18). Accordingly, the Bax:Bcl-2 ratio decreased (0.71 ± 0.32, p = 0.02) in the favor of anti-apoptotic Bcl-2 in SSc fibroblasts (Figure 1A). At the protein level, Bcl-2 expression was 1.6 ± 0.17-fold more than its expression in normal fibroblasts and the Bax:Bcl2 ratio decreased by 2.11 ± 0.26-fold (p < 0.05) compared to those of normal (Figure 1B). The results obtained from real-time PCR and Western blot confirmed the elevated level of anti-apoptotic protein Bcl-2 and decreased ratio of Bax:Bcl-2 in SSc fibroblasts confirming the fact that SSc fibroblasts tend to survive.

Modulation of the expression of the Bcl-2 and Bax mRNAs by miR-29a

To investigate the correlation between miR-29a level and the relative expression of Bcl-2 and Bax mRNAs, SSc fibroblasts were transiently transfected with miR-29a mimic, inhibitor or scrambled RNA (Figure 2A). Real-time PCR data analysis showed that miR-29a mimic led to a decrease in Bcl-2 expression by 2.3 ± 0.85-fold (p = 0.0005) in SSc fibroblasts, whereas Bax expression did not changed significantly (p = 0.32). In SSc fibroblasts, decline in Bcl-2 mRNA levels, in response to miR-29a mimic, increased the Bax:Bcl-2 ratio by 3.6 ± 1.8-fold (p = 0.05) (Figure 2A). It should be noted that inhibition of miR-29a did not change the Bcl-2 expression and hence the Bax:Bcl-2 expression ratio.

Considering the fact that TGF-β plays a key role in SSc and probably other fibrotic diseases, the normal fibroblasts were treated with TGF-β (10 ng/ml) for 48 h to induce fibrotic fibroblasts, as indicated in previous studies [28,35]. The effect of miR-29a on Bcl-2 and Bax expression and Bax:Bcl-2 expression ratio was then investigated in normal and TGF-β-treated fibroblasts (Figure 2B). In the presence of TGF-β, fibroblasts showed an increase in Bcl-2 expression level (1.94 ± 0.55-fold, p = 0.002) similar to SSc fibroblasts. Expectedly, miR-29a mimic overturned the positive effects of TGF-β and lowered the Bcl-2 mRNA expression (−1.6 ± 0.32-fold, p = 0.01). Normal fibroblasts also showed decline in Bcl-2 expression in the presence of miR-29a mimic (−2.01 ± 0.30-fold, p = 0.004). The results showed that Bax mRNA expression level significantly increased in normal fibroblasts after both TGF-β (p = 0.04) and miR-29a (p = 0.005) treatments. MiR-29a mimic played an opposing effect on Bax and Bcl-2 expression levels by increasing the Bax:Bcl-2 ratio in both TGF-β-treated (3.5 ± 1.8-fold, p = 0.03) and normal (4.7 ± 2.4-fold, p = 0.01) fibroblasts while enhancing their susceptibilities to apoptosis. Inhibition of miR-29a, by inhibitor, demonstrated no effect on Bcl-2 and Bax mRNA expression.

Modulation of Bcl-2 and Bax proteins by miR-29a

Antibodies were applied against Bcl-2 and Bax in order to follow any changes in their protein levels in response to miR-29a using Western-blot analysis. Increased expression of miR-29a concomitant with decreased expression of Bcl-2 protein (−1.6 ± 0.1, p < 0.05) indicated that miR-29a has successfully inhibited its target production. MiR-29a mimic resulted in more enhancement of the Bax:Bcl-2 ratio (1.84 ± 0.15, p < 0.05) in SSc fibroblasts (Figure 3A). In normal and TGF-β-stimulated fibroblasts, miR-29a mimic
was sufficient to decrease Bcl-2 expression level (−1.6 ± 0.02, \( p < 0.05 \)), whereas TGF-β increased Bcl-2 after inhibiting miR-29a (1.5 ± 0.02, \( p < 0.05 \)) (Figure 3B). It should be noted that expression of Bax protein level was not significant during TGF-β (\( p = 0.18 \)) and miR-29a mimic (\( p = 0.58 \)) treatments. As a result, the Bax:Bcl-2 expression ratio was associated with Bcl-2 differential expression. Collectively, data analysis of Western blots revealed similar results to those obtained via real-time PCR. MiR-29a mimic was sufficient to decrease the anti-apoptotic Bcl-2 expression level and increase the Bax:Bcl-2 ratio and it consequently led to the enhancement of fibroblasts susceptibility to apoptosis.

miR-29a mimic modulates the expression of Bcl-XL and TNF-α

In addition to Bax and Bcl-2, we also analyzed the expression of Bcl-XL, an anti-apoptotic protein, in SSc and normal fibroblasts in response to miR-29a treatment (Figure 4). The basal level of Bcl-XL in SSc fibroblasts was higher (\( p = 0.04 \)) than that of normal fibroblasts. Restoration of miR-29a expression decreased the elevated level of Bcl-XL mRNA (\( p = 0.0001 \)) in SSc fibroblasts. Moreover, over-expression of miR-29a in TGF-β-stimulated and normal fibroblasts significantly decreased Bcl-XL mRNA level (\( p = 0.006, p = 0.008 \)) in these cells (Figure 4A). At protein level, miR-29a decreased Bcl-XL expression in SSc and TGF-β-stimulated fibroblasts except that miR-29a had no significant effect on Bcl-XL expression in normal cells (Figure 4B).

We also verified the expression of TNF-α, a pro-inflammatory cytokine which is expressed by dermal fibroblasts in response to inflammation and indicating fibroblasts contribute to chronic inflammation. We showed that the basal level of TNF-α in SSc fibroblasts is higher than that of normal cells. Moreover, miR-29a restoration had no effect on mRNA level of TNF-α in normal skin fibroblasts; however, it significantly (\( p = 0.01 \)) decreased TNF-α mRNA in SSc fibroblasts (Figure 4A).

miR-29a mimic induces apoptosis in SSc and TGF-β-stimulated fibroblasts

Then, to elucidate the apoptotic effect of miR-29a, annexin-V and Hoechst 33258 staining assay were followed (Figure 5). Fibroblasts were transfected with miR-29a mimic for 24 h and stained with annexin-V-FITC and propidium iodide (PI) to investigate apoptosis by flow cytometry. Annexin V is a probe that is conjugated with the fluorescence dye fluorescein isothiocyanate (FITC) to detect phosphatidylinerine (PS) on the outer membrane of the apoptotic cells, and PI is a DNA
binding fluorochrome for detecting apoptotic and necrotic cells. According to fluorescence activated cell sorting (FACS) data, apoptotic cells were detected in upper left [annexin-V-FITC (+) PI (-)] and right [annexin-V-FITC (+), PI (+)] quadrants (Figure 5A). Total percent of apoptotic cells were calculated by the sum of early and late apoptotic cells (upper left and right quadrants) (Figure 5B). The apoptosis rates in normal, TGF-β-treated and SSc fibroblasts were 1.9 ± 0.41, 4.3 ± 0.83 and 1.9 ± 0.35, respectively. Exogenous restoration of miR-29a level in SSc and TGF-β-treated fibroblasts caused a significant population of cells (27.1 ± 8.22%, p = 0.0065 and 24.87 ± 4.87%, p = 0.005, respectively) to enter apoptosis. We also detected apoptosis in normal fibroblasts after over-expression of miR-29a (9.21 ± 0.43%) indicating that an increase in the basal level of miR-29a could be apoptotic for dermal fibroblasts. (Figure 5C). Apoptotic cell percent was estimated by counting 300 cells per sample (n = 4) (Figure 5D).

Discussion

There are numerous triggering factors and pathophysiological processes contributing to fibrosis and deregulation of apoptosis. Increased population and prolonged presence of activated fibroblasts along with excessive amount of ECM are common consequences seen in fibrotic disorders [36]. Regarding the fact that pathologic changes in fibroblasts play a main role in development of fibrosis, recent anti-fibrotic remedial strategies have focused on fibroblast-specific approaches. It seems that overcoming fibroblasts resistance to apoptosis is an idoneous way to prevent development of fibrosis in the skin and other fibrotic tissues. A growing number of reports have confirmed the relationship between miR-29 family and development of fibrosis in the skin and also in internal organs, including lung [29], liver [37], heart [38] and kidney [30]. Moreover, miR-29a expression is negatively regulated by the well-known pro-fibrotic factor, TGF-β, in SSc and other fibrotic diseases [28,30,39].
It has been predicted that miR-29s directly targets about 20 collagen mRNAs in addition to mRNAs for other ECM components [40]. Well-established models for different kinds of fibrosis have confirmed that restoration of miR-29s attenuates the expression of fibrotic markers [28,31,41]. These findings highlight the therapeutic potential of miR-29s in fibrosis [42]. MiR-29s expression level, especially that of miR-29a, is down-regulated in SSc [42]. It has been reported that over-expression of miR-29a in SSc fibroblasts exerts counter-effect in deregulating secretion of ECM components, especially production of collagen types I and III [28].

Many pathological factors trigger Bcl-2 family proteins to deregulate apoptosis [43]. Many apoptotic signals converge on mitochondria and promote the protective balance between pro- and anti-apoptotic members of the Bcl-2 family, which disturbs MOMP and results in cytochrome c release into the cytoplasm [44,45]. Tumor cells in more than half of cancers use over-expression of Bcl-2 or Bcl-XL to suppress apoptosis [16,46,47]. Therefore, different strategies, including repressing Bcl-2 and Bcl-XL transcription or their mRNA translation and usage of drugs antagonist to Bcl-2, have been developed for cancer therapy [48–50]. Currently, antisense oligonucleotides raised against Bcl-2 mRNA are introduced to phases II and III clinical trials in different cancers [16,48].

The importance of Bcl-2 family, especially Bcl-2 and Bcl-XL, in programmed death of fibroblasts has been

Figure 3. Expression of Bcl-2 and Bax proteins after over-expression and inhibition of miR-29a. The expression of genes and Bax:Bcl-2 ratio in scrambled RNA treated cells were defined as 1 or 100 and other expression results were compared to it. (A) SSc fibroblasts (n = 10) were transfected with scrambled RNA (10 nM), miR-29a mimic (10 nM) or miR-29a inhibitor (50 nM) for 24 h. MiR-29a over-expression decreased Bcl-2 expression and enhanced the ratio. (B) Normal fibroblasts transfected with miR-29a mimic, inhibitor or scrambled RNA for 24 h. Then starved and treated with TGF-β (10 ng/ml) (n = 10) for 48 h. TGF-β as a fibrotic factor increased Bcl-2 expression and its effect was reversed significantly (p < 0.05) with over-expression of miR-29a. (C) Representative immunoblots of control and patient. All the values are mean ± SEM. *p < 0.05. n: number of samples.
confirmed in liver and lung fibrosis [49–51]. In the present study, we did not detect apoptosis in SSc fibroblasts and, therefore, it was proposed that it may be associated with the increased expression of Bcl-2 and Bcl-XL in these cells. In addition, the observed decline in the Bax:Bcl-2 expression ratio may be related to the overall tendency of SSc fibroblasts to survive. Evidence in cancer research indicates that miR-29a regulates apoptosis by targeting different points in the apoptosis pathway. Xiong et al. [34] generated pc3-gab-BCL2 expression vector and defined the BCL-2 3'-untranslated region (3'-UTR) as the direct target of miR-29a. They reported that miR-29a suppresses Bcl-2 expression and promotes apoptosis via induction of MOMP and cytochrome c release in cytoplasm [34]. Considering that Bcl-2 is the direct target of miR-29a, this study clearly showed that miR-29a decreased Bcl-2 expression and consequently increased the ratio of Bax:Bcl-2, and finally triggered apoptosis in SSc fibroblasts (Figure 5). We next examined the influence of miR-29a on apoptosis of TGF-β-stimulated fibroblasts. TGF-β is the most potent factor to induce fibrotic phenotype [7] and protect fibrotic fibroblasts from apoptosis [52,53]. MiR-29a reversed the anti-apoptotic effect of TGF-β and induced apoptosis in fibroblasts; it is worth to mention that miR-29a may induce apoptosis in other fibrotic disease.

There are limited reports on the apoptosis of SSc fibroblasts. Santiago et al. used TUNEL assay and did not detect cell death in dermal fibroblasts of SSc lesions and normal skin. They further showed that SSc fibroblasts are resistance to Fas-induced apoptosis. They concluded that Bcl-2, Bcl-XL and Bax proteins modulate decreased susceptibility to Fas-induced apoptosis [54]. We found the expression of TNF-α, an inducer of apoptosis is higher in SSc fibroblasts compared to normal fibroblasts. Although there is not a definitive study investigating the apoptotic effect of TNF-α on SSc fibroblasts, the apoptotic effect of TNF-α on dermal fibroblasts led to the conclusion that SSc fibroblasts are resistance to TNF-α-induced apoptosis. Moreover, TNF-α has been previously considered as an anti-fibrotic cytokine suppressing collagen expression [55,56]. However, Voloshenyuk et al. [57] suggested that TNF-α contributes
to collagen cross-linking, which determines the stability of the ECM. Furthermore, the positive role of TNF-α in both TGF-β [58] and ECM production [59,60] has introduced TNF-α as a fibrotic factor, and the inhibition of TNF-α has been suggested as a therapeutic strategy against lung and cardiac fibrosis [58,59]. There is a negative relation between miR-29a and TNF-α in dendritic cells [61]; it seems that miR-29a affects inflammatory responses through decrease in TNF-α.

Figure 5. Apoptosis assays. (A) Flow cytometry assay. SSc, TGF-β-stimulated and normal fibroblasts in the presence or absence of miR-29a mimic were stained with annexin-V-fluorescein and propidium iodide (PI) to detect early (annexin-V-FITC, upper left quadrant) and late (annexin-V-fluorescein + PI upper right quadrant) apoptosis. The lower left quadrant represents live cells. (B) Flow cytometry quantification of apoptosis (n = 4). All the values are mean ± SEM. (C) Apoptosis phenotype was detected using fluorescent DNA dye Hoechst 33258 and observed under a fluorescent microscopy (×20). Influence of miR-29a on apoptosis was detected from condensed or fragmented chromatin. The nuclei of apoptotic cells are bright blue ones which are distinguished from light blue color of live cells. (D) Apoptotic rate (AR) was estimated by counting 300 nuclei per sample. All the values are mean ± SEM. *p < 0.05. NF, normal fibroblast; TF, TGF-β-treated fibroblast; SF, SSc fibroblast; n, number of samples.
expression. Here, we showed that the high level of TNF-α in SSc fibroblasts significantly \((p = 0.01)\) subsided following the treatment with miR-29a.

Collectively, we conclude that in addition to regulating aberrant expression of ECM, miR-29a has a broader role in fibrosis cascade. We showed that miR-29a clearly disturbed the pathogenic balance between the anti- and pro-apoptotic proteins (Bcl-2, Bcl-XL and Bax), and also induced apoptosis in SSc and TGF-β-stimulated fibroblasts. Recently, many anti-fibrotic treatment strategies have focused on miRNAs, as epigenetic regulators, to improve fibrotic phenotype in affected tissues. We provide the first evidence of apoptotic-inducing property of miR-29a in fibrotic fibroblasts which offers novel benefits for the increased interests toward the development of fibroblast-specific anti-fibrotic therapies.

**Acknowledgements**

We appreciate the assistance of the Iran Rheumatology Center for selecting our patients.

**Declaration of interest**

The authors have declared no conflicts of interest. This work was generously supported by grants from the Iran National Science Foundation (INSF) and Tehran University of Medical Science (TUMS).

**References**


Supplementary material available online
Supplementary materials 1 and 2.