Normal skin and hypertrophic scar fibroblasts differentially regulate collagen and fibronectin expression as well as mitochondrial membrane potential in response to basic fibroblast growth factor

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Abstract

Basic fibroblast growth factor (bFGF) regulates skin wound healing; however, the underlying mechanism remains to be defined. In the present study, we determined the effects of bFGF on the regulation of cell growth as well as collagen and fibronectin expression in fibroblasts from normal human skin and from hypertrophic scars. We then explored the involvement of mitochondria in mediating bFGF-induced effects on the fibroblasts. We isolated and cultivated normal and hypertrophic scar fibroblasts from tissue biopsies of patients who underwent plastic surgery for repairing hypertrophic scars. The fibroblasts were then treated with different concentrations of bFGF (ranging from 0.1 to 1000 ng/mL). The growth of hypertrophic scar fibroblasts became slower with selective inhibition of type I collagen production after exposure to bFGF. However, type III collagen expression was affected in both normal and hypertrophic scar fibroblasts. Moreover, fibronectin expression in the normal fibroblasts was up-regulated after bFGF treatment. bFGF (1000 ng/mL) also induced mitochondrial depolarization in hypertrophic scar fibroblasts (P < 0.01). The cellular ATP level decreased in hypertrophic scar fibroblasts (P < 0.05), while it increased in the normal fibroblasts following treatment with bFGF (P < 0.01). These data suggest that bFGF has differential effects and mechanisms on fibroblasts of the normal skin and hypertrophic scars, indicating that bFGF may play a role in the early phase of skin wound healing and post-burn scar formation.

Key words: bFGF; Hypertrophic scar; Fibroblast; Extracellular matrix; Mitochondria; Gene expression

Introduction

Wound healing is a dynamic and closely interactive process of various growth factors, fibroblasts, and the formation of new blood vessels and extracellular matrices. Molecularly, the growth factors or cytokines derived from monocyte/macrophage or lymphocytes are essential to attract different types of cells to move into the wound area. Fibroblasts play an important role in wound healing by producing a provisional wound healing matrix, including collagen and fibronectin (1). Some fibroblasts differentiate into myofibroblasts, which are principally responsible for tissue contraction, but also produce extracellular matrix components. In normal human wound healing, once tissue integrity has been restored, the
myofibroblasts disappear from the scar (2). However, in pathological situations such as hypertrophic scar (HS), myofibroblasts persist in the tissue (3). In particular, increased activity of fibrogenic cytokines [e.g., transforming growth factor β1 (TGF-β1), insulin-like growth factor 1 (IGF-1), and interleukin-1] and exaggerated responses to these cytokines may also play a role in post-burn scars such as fibrosis and keloids (3). The aberrant fibroblast phenotype also seems to contribute to the hypertrophic scars or post-burn scars, which may be due to the differential response of normal and HS fibroblasts to various growth factors. Specific growth factors targeted would include TGF-β1, IGF-1, or basic fibroblast growth factor (bFGF) (4,5). It has been shown that bFGF is also a potent chemotactic factor for fibroblasts and endothelial cells, it can promote or inhibit cell differentiation, and it also functions as a potent angiogenic and neurotrophic factor (6,7). Indeed, previous in vitro and in vivo studies have demonstrated that bFGF treatment effectively regulates granulation, tissue formation through apoptosis and inhibition of collagen synthesis and, therefore, can promote scarless repair (8,9). The scar-inhibition effect of bFGF on sutured wounds was confirmed clinically, i.e., when bFGF was injected into the dermis or topically applied after surgery, no hypertrophic scars formed later. In many cases, there was only a fine linear scar (10). However, the molecular mechanism underlying the action of bFGF in reducing the hypertrophic scar and enhancing wound healing remains to be demonstrated.

In the current study, we determined the role of bFGF during the regulation of type I and III collagen and fibronectin expression in fibroblasts derived from human normal skin and from hypertrophic scar biopsies. These proteins are structural molecules of the provisional matrix in wound repair. In addition, mitochondria may be an important mediator in extracellular matrix homeostasis and cell proliferation (11-13). Therefore, the mitochondrial membrane potential and cellular ATP level were measured before and after bFGF treatment of these fibroblasts. Our study provides evidence that mitochondria mediate the reversible regulation of bFGF capacity in wound healing and uncontrolled scar formation.

**Material and Methods**

**Cell cultures and treatments**

Fibroblasts were isolated from human skin biopsies obtained from five patients who underwent plastic surgery to correct excess scar tissue. The laboratory protocol was approved by the medical and ethics committee of Southern Medical University and the People’s Hospital of Guangdong Province and written informed consent was obtained from the patients. Fibroblasts were isolated from the dermis of the biopsies according to a method previously described (14,15). The cells, at 70-80% confluence, were treated with different concentrations of bFGF in a serum-free medium.

**Transmission electron microscopy**

Ultrastructural analysis of fibroblasts was conducted by transmission electron microscopy. Cells were fixed for Epon-Araldite embedding in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and then post-fixed in 1% osmium tetroxide, pH 7.4, followed by ethanol dehydration. Ultrathin sections were stained on-grid with 5% uranyl acetate and lead citrate, and examined with an electron microscope (Philip CM10, Holland).

**Measurement of fibroblast proliferation and death**

The fibroblasts were grown and treated or not with bFGF for 72 h before analysis. Cells were trypsinized with phosphate-buffered trypsin/EDTA at 37°C for 10 min, and collected by centrifugation. The number of released cells was quantified by counting in a hemocytometer chamber, with viability assessed by 0.4% trypan blue exclusion. The assay was repeated three times.

**Type I and III collagen and fibronectin assays**

Type I and III collagen and fibronectin levels in the conditioned culture media of human skin fibroblast cultures were measured using a commercially available ELISA kit according to manufacturer instructions (Adlitteram Diagnostic Laboratories Inc., China). The levels of type I and III collagen and fibronectin in the conditioned culture media were assayed in triplicate and measured with an automatic microplate reader (SpectraMax M5, USA) at a wavelength of 450 nm.

**RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)**

The fibroblasts were grown and treated with bFGF for 72 h, and total RNA was extracted from the cells and converted into cDNA using an E.Z.N.A. total RNA kit (Omega Bio-tek, USA). The fibroblasts were further subjected to RT-PCR amplification of gene expression. A PrimeScript™ RT-PCR kit (TaKaRa, Japan) was used according to manufacturer instructions (16). The following oligonucleotide primer sets (Invitrogen, USA) were designed and synthesized: type I collagen forward: 5’-CATGCCGTGACTTGAGACTCA-3’ and reverse: 5’-AGGCCCATGAAGCAGTT-3’, producing a 292-bp band; type III collagen forward: 5’-CGAGCTTCCCAGAACGCTACA-3’ and reverse: 5’-TTCGTGCAACCATCC
TCCA-3', producing a 170-bp band; fibronectin forward: 5'-TTCCCTTGCTGGTATCATGGCA-3' and reverse: 5'-TATTCCGG TTTCCGTTTCCA-3', producing a 156-bp band, and β-actin forward: 5'-GCCCTGAGGCACTCTTTCCA-3' and reverse: 5'-GCGGATGTCCACGTCACA-3', producing a 101-bp band. The reaction mixtures were denatured at 95°C for 10 min, and then amplified for 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min and later stored at 4°C. The PCR products were then electrophoresed in a 2% agarose gel. The expression levels of type I and III collagen and fibronectin were quantified and normalized with β-actin using densitometry.

Determination of mitochondrial membrane potential
Mitochondrial membrane potential (ΔΨm) was measured using the fluorescent probe JC-1. This dye normally exists in the mitochondrial matrix as a dimeric configuration emitting a red fluorescence, but then assumes a monomer configuration, emitting a green fluorescence when ΔΨm depolarizes. The fibroblasts were grown and treated with bFGF for 72 h and trypsinized and 10^5 fibroblasts were collected by centrifugation (10 min, 1000 rpm). After washing twice with PBS, the cells were incubated with 2 µM JC-1 dye for 15 min at 37°C in a humidified incubator. JC-1 was excited at 488 nm and the aggregate signal (red) was analyzed at 590 nm (FL2). The monomer signal (green) was analyzed at 525 nm (FL1) on a FACScan instrument (Becton Dickinson, USA) using the Cell Quest software (Becton Dickinson). The ratio of the mean red to the mean green JC-1 fluorescence was calculated as a relative mitochondrial membrane potential as described in a previous study (17).

Cytosolic ATP measurements
Relative levels of cellular ATP were assayed using the CellTiter 96 assay kit from Promega (country) according to the manufacturer’s instructions (18). Briefly, 5 x 10^3 cells were plated onto a 96-well plate (BD Biosciences, country) and grown for 72 h. The cells were then mixed with 0.1 mL of the CellTiter substrate for 10 min and the luminescent output was read with an automatic microplate reader (SpectraMax M5). The cellular ATP correlations are reported as percent luminescence at each drug concentration in relation to the luminescence of untreated normal cells.

Statistical analysis
Data are reported as mean ± SEM and were analyzed by the Student paired t-test and one-way ANOVA using SPSS 13.0 for Windows. P ≤ 0.05 was considered to be statistically significant.

Results
Ultrastructural analysis of the two types of fibroblasts from normal skin and hypertrophic scars
To determine whether the fibroblasts isolated from hypertrophic scars were phenotypically distinct from normal human skin fibroblasts, we examined their ultrastructure by electron microscopy. Fibroblasts from hypertrophic scars have a myofibroblast-like structure with a fibronexus (Figure 1B), a subplasmalemmal complex involving intracellular actin microfilaments (Figure 1C), and an external lamina. However, this structure was not observed in fibroblasts from normal human skin (Figure 1A). Moreover, more than 80% of the HS fibroblasts also showed myofibroblast differentiation under electron microscopy, i.e., numerous microfilament bundles parallel to the main axis of the cell, dense plaques at the plasma membrane, and/or the external lamina surrounding the cells (data not shown).

Effect of bFGF on fibroblast proliferation and death
To elucidate the effects of bFGF on the growth of the two types of fibroblasts, the fibroblasts were treated with or without bFGF, 0.1-1000 ng/mL, for 72 h and then counted. In the absence of bFGF, the mean number of HS fibroblasts was 19.43 ± 1.27 x 10^5, and the number of normal fibroblasts was 9.57 ± 0.78 x 10^5 (P < 0.05). In the presence of 100 ng/mL bFGF, the mean number of normal fibroblasts increased to a peak of 58.17 ± 3.75 x 10^5 (P < 0.01). In the presence of 100 ng/mL bFGF, the mean number of HS fibroblasts increased to a peak of 41.50 ± 5.67 x 10^5 (P < 0.01). However, in the presence of 1000 ng/mL bFGF, the mean number of HS fibroblasts was reduced to 10.20 ± 0.79 x 10^5 (P < 0.01), while that of normal fibroblasts was 30.13 ± 3.90 x 10^5 (P < 0.01; Figure 2A). Therefore, 1000 ng/mL bFGF reduced HS fibroblast proliferation by approximately 48%. Furthermore, the number of trypan blue-positive dead cells increased significantly in cultures treated with 1000 ng/mL bFGF for 72 h (P < 0.01; Figure 2C). However, there was no significant difference in the number of dead cells in the normal fibroblast group (Figure 2B).

Effect of bFGF on fibroblast collagen production
We determined the effect of bFGF on the regulation of collagen expression in both normal and HS fibroblasts. Type I collagen produced by HS fibroblasts was 0.031 ± 0.004 µg·mL⁻¹·mg protein⁻¹, which was significantly higher than that
produced by normal fibroblasts (0.017 ± 0.004 µg·mL⁻¹·mg protein⁻¹) in the absence of bFGF culture (P < 0.05). However, in the presence of 1, 10, 100, and 1000 ng/mL bFGF, type I collagen in HS fibroblasts significantly decreased to 0.015 ± 0.008, 0.014 ± 0.006, 0.016 ± 0.005, and 0.015 ± 0.005 µg/mL, respectively (P < 0.01). In contrast, after exposure to different concentrations of bFGF, the levels of expression of type I collagen were not significantly changed in normal fibroblasts (Figure 3). Expression of type III collagen was not significantly altered by bFGF treatment in either normal or HS fibroblasts (Figure 3).

**Effect of bFGF on fibroblast collagen mRNA expression**

Next, we also determined the effect of bFGF on the expression of collagen mRNA levels in these fibroblasts before and after bFGF treatment by semi-quantitative RT-PCR. As shown in Figure 4, the expression of type I collagen mRNA in HS fibroblasts was significantly higher than that in the normal fibroblasts in the absence of bFGF (P < 0.05). However, in the presence of 1, 10, 100, and 1000 ng/mL bFGF, the expression of type I collagen mRNA significantly decreased to 62, 60, 61, and 69% of the pretreated value, respectively (P < 0.01). In contrast, no significant changes were observed in normal fibroblasts after treatment with bFGF (Figure 4). In addition, the expression of type III collagen mRNA was not significantly changed after bFGF treatment (Figure 4).

**Effect of bFGF on fibroblast fibronectin expression**

As shown in Figures 3 and 4, the effects of bFGF on fibronectin mRNA and protein levels were also assessed in normal and HS fibroblasts. In the presence of bFGF, normal fibroblasts showed an increase in the expression of fibronectin protein (P < 0.01; Figure 3) and mRNA (P < 0.01; Figure 4), whereas there was no significant difference in fibronectin expression in HS fibroblasts (Figures 3 and 4). The comparison of the generalized estimating equations between the two types of fibroblasts showed a statistically significant difference in fibronectin expression (P < 0.05).

**Effect of bFGF on the mitochondrial membrane potential and cellular ATP levels**

Previous studies have demonstrated that mitochondrial function plays an important role in the function and survival of fibroblasts for cell repair, growth, and extracellular matrix production during wound healing (19,20). We, therefore, examined the changes in mitochondrial function after treating the two types of fibroblasts with bFGF by detecting changes in mitochondrial membrane potential and in cellular ATP levels. We observed that changes in the ratio of JC-1 red to green fluorescence in HS fibroblasts decreased after bFGF treatment (Figure 5A). The relative mitochondrial membrane potential of HS fibroblasts significantly decreased from a pretreatment level of 261.14 ± 138.29 to 81.68 ± 44.80% after treatment with 1000 ng/mL bFGF (P < 0.01; Figure 5B), suggesting that the mitochondrial depolarization of HS fibroblasts was induced by bFGF. bFGF treatment (10, 100 and 1000 ng/mL) also induced a significant increase in cellular ATP levels in normal fibroblasts (P < 0.01). However, in the presence of 1000 ng/mL bFGF, intracellular ATP levels were 38.16% lower than the pretreated level in HS fibroblasts (P < 0.05; Figure 6).

**Discussion**

The process of fibrosis and normal wound healing is closely associated with fibroblast growth and collagen formation. Excessive myofibroblast differentiation and extracellular matrix formation may be involved in hypertrophic scarring and formation of post-burn scars, such as fibrosis and keloids (3), resulting in hypertrophic scars, the management of which remains a challenge in clinical practice. However, there may be phenotypic differences between fibroblasts from normal skin and from hypertrophic scars in response to cytokines. bFGF, a self-limited and potent wound-healing agent, is being widely used for research in this field (9,10,21), although the underlying molecular mechanism remains to be determined. It has been reported that mitochondria are related to myofibroblast differentiation (22). In the present study, we determined differential regulation of growth and collagen and fibronectin synthesis as well as mitochondrial membrane potential in normal and HS fibroblasts after treatment with bFGF.

We observed that production of type I collagen by HS fibroblasts was much higher than production by normal skin fibroblasts in the absence of bFGF, while production of type I collagen by HS fibroblasts was significantly inhibited by a high dose of bFGF. A previous study revealed that excessive production and deposition of type I collagen played an important role in hypertrophic scar formation (23). In this respect, a previous study showed that bFGF was able to reduce type I collagen levels in keloid fibroblasts as detected by immunostaining (8). The reduction of type I collagen production was also observed under immunofluorescent and scanning electron microscopy after administration of bFGF for 6 weeks (24). The inhibition was associated with transcriptional down-regulation of type I collagen gene expression (8,25). Our data are in agreement with these previous data. Furthermore, although the rate of type III collagen synthesis is increased in the early wound healing period (26,27), our data showed that this type of collagen might not be involved in bFGF-mediated
wound healing. Fibronectin is also highly up-regulated after wound healing and acts as a provisional matrix, promoting fibroblast migration in the early phase of wound healing (28,29). Excessive deposition of fibronectin may contribute, at least in part, to hypertrophic scar formation (30). The present experiments indicated that bFGF increased fibronectin production, possibly by the up-regulation of fibronectin gene expression in normal fibroblasts, but this did not occur in HS fibroblasts. Therefore, these data suggest that bFGF-mediated production of extracellular matrix and gene expression were cell phenotype-dependent. In addition, identification of bFGF in the inhibition of type I collagen expression in HS fibroblasts and the increase of fibronectin in normal fibroblasts provide insightful information regarding the potential mechanism of action of bFGF in the early phase of wound healing and in post-burn scar formation.

Previously reported in vivo data showed that the early subcutaneous administration of bFGF inhibited the differentiation of fibroblasts and excessive formation of myofibroblasts in wound healing (8). It was recently reported that bFGF-induced granulation tissue formation was due to the increase in cell proliferation that exceeded cell apoptosis on days 4 and 7; however, suppression of granulation tissue formation was mainly due to the increased fibroblast apoptosis on day 14 (10). The present data suggest that growth of HS fibroblasts became slower than that of normal fibroblasts after exposure to bFGF. Taken together, these data suggest an explanation for the difference in response to bFGF by normal fibroblasts and HS fibroblasts.

Mitochondria are perceived to be necessary for controlling cell growth, differentiation, death, gene expression and other key cellular processes (31). Wound healing is a process that uses cell energy to move the wound cells and to form the extracellular matrix. For example, fibroblasts can survive but not replicate or synthesize collagen in a low energy environment (32). It is evident that mitochondrial functioning varies with the changes in the extracellular matrix composition to regulate the extracellular matrix. Transforming growth factor-β1 has been recently demonstrated to be able to modify extracellular matrix composition and structure and to affect mitochondrial function (20). As a result, knowledge of the differences in mitochondria between normal fibroblasts and HS fibroblasts treated with bFGF would be important in determining how bFGF regulates the scarless wound repair process. However, there are no previous data documenting the differences in mitochondrial functions between normal fibroblasts and HS fibroblasts after treatment with bFGF. In the present study, it was observed that bFGF had no effect on the modulation of the ΔΨm in normal skin fibroblasts, but led to depolarized mitochondria in HS fibroblasts. Maintaining mitochondrial membrane potential was found to be a prominent mechanism in the production of cellular ATP by means of the mitochondria (33,34). Consistent with this mechanism, our results showed that bFGF induced an increase of relative ATP levels in normal fibroblasts and a decrease in cellular ATP in HS fibroblasts, which might contribute to the different growth rate between normal fibroblasts and HS fibroblasts. Interestingly, a high concentration of bFGF (1000 ng/mL in the current study) led to significant dissipation of ΔΨm and HS fibroblast death, but did not affect normal fibroblasts. It has been previously demonstrated that mitochondrial permeability transition causes ΔΨm dissipation, uncoupling of oxidative phosphorylation, ATP depletion, and equilibration of small solutes and ions between the cytosol and the mitochondrial matrix to control cell death or survival (31). However, this matter needs to be further investigated regarding whether bFGF-induced death of HS fibroblasts is due to changes in mitochondrial function or due to bFGF-induced myofibroblast death for eliminating this kind of myofibroblast-differentiated fibroblast in pathological tissue contractures. Data from the current study suggest that the effect of bFGF on skin fibroblasts may occur through changes in mitochondrial function, indicating that future studies could target bFGF for the control of wound healing and for antifibrosis therapy.

Acknowledgments

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References

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Figure 1. Ultrastructure of normal skin and hypertrophic scar (HS) fibroblasts. A, The normal fibroblast showed the absence of myo-fibroblast-like features (arrowhead). B, Transmission electron micrograph of a cross-section of HS fibroblasts showed fibroblast-to-myofibroblast transdifferentiation according to features that are similar to myofibroblasts, with the external lamina (arrowhead) and with hemidesmosome-like structure corresponding to the so-called fibronexus (arrows). C, The characteristics of actin microfilament bundles and the subplasmalemmal dense plaque (arrow) in another transformed HS fibroblast. Bars: A and B = 1 μm; C = 0.5 μm.
Figure 2. Effect of basic fibroblast growth factor (bFGF) on proliferation and death of normal skin and hypertrophic scar (HS) fibroblasts. A, The proliferation of HS fibroblasts was reduced compared to that of normal fibroblasts following treatment with bFGF at concentrations of 0.1 to 1000 ng/mL for 72 h. B, bFGF treatment did not affect the mortality of normal fibroblasts. C, bFGF treatment resulted in an increase of HS fibroblast mortality. Data are reported as means ± SEM. The experiments were performed in triplicate and repeated at least once. **P < 0.01 vs untreated fibroblasts; #P < 0.05, ##P < 0.01 vs normal skin fibroblasts (paired t-test and one-way randomized ANOVA).
Figure 3. Effect of basic fibroblast growth factor (bFGF) on type I/III collagen and fibronectin production by normal and hypertrophic scar (HS) fibroblasts. The cells were grown and treated with bFGF at concentrations of 0.1 to 1000 ng/mL for 72 h and subjected to ELISA. bFGF decreased type I collagen production by HS fibroblasts and increased fibronectin production by normal fibroblasts. Data are reported as means ± SEM. The experiments were carried out in triplicate and repeated at least once. *P < 0.01 vs untreated fibroblasts; # P < 0.05, ## P < 0.01 vs normal skin fibroblasts (paired t-test and one-way randomized ANOVA).

Figure 4. Effect of basic fibroblast growth factor (bFGF) on the expression of type I/III collagen and fibronectin mRNA in normal and hypertrophic scar (HS) fibroblasts. The cells were grown and treated with bFGF at concentrations of 0.1 to 1000 ng/mL for 72 h and then subjected to RT-PCR analysis of type I (A), type III collagen (B), and fibronectin (C) expression. bFGF down-regulated type I collagen mRNA in HS fibroblasts and up-regulated fibronectin mRNA in normal fibroblasts. Data were normalized to a β-actin loading control. The bottom graphs showed the quantitative RT-PCR data. Data are reported as means ± SEM. *P < 0.05, **P < 0.01 vs untreated fibroblasts; #P < 0.05, ##P < 0.01 vs normal skin fibroblasts (paired t-test and one-way randomized ANOVA).
Figure 5. Flow cytometry analysis of the mitochondrial membrane potential in normal and hypertrophic scar (HS) fibroblasts. Cells were grown and treated with basic fibroblast growth factor (bFGF) at concentrations of 0.1 to 1000 ng/mL for 72 h and then subjected to JC-1 staining and flow cytometry. The relative mitochondrial membrane potential was expressed as the ratio of JC-1 red fluorescence (upper quadrant) to green fluorescence (lower right quadrant) in the normal (A, top) and HS fibroblasts (A, bottom). bFGF depolarized HS fibroblasts mitochondria, with no effect on mitochondrial membrane potential in normal fibroblasts. The quantitative data (B) are reported as means ± SEM. ** P < 0.01 vs untreated fibroblasts (paired t-test and one-way randomized ANOVA).
Figure 6. Effect of basic fibroblast growth factor (bFGF) on the regulation of cellular ATP levels in normal and hypertrophic scar (HS) fibroblasts. Cells were grown and treated with bFGF at concentrations of 0.1 to 1000 ng/mL for 72 h and then subjected to the luciferin-luciferase assay. bFGF decreased the intracellular ATP levels in HS fibroblasts and increased the ATP levels in the normal fibroblasts. Data are reported as means ± SEM. *P < 0.05, **P < 0.01 vs untreated fibroblasts (paired t-test and one-way randomized ANOVA).