Effects of basic fibroblast growth factor on expressions of collagen and fibronectin in normal skin and hypertrophic scar fibroblasts**☆◆

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Abstract

BACKGROUND: Basic fibroblast growth factor (bFGF) can promote production of collagen, fibronectin and matrix enzyme in healing wounds. However, dysregulation of this process, such as the abnormal coordination of cell proliferation, extracellular matrix and neovascularization formation, or remodeling of the wound matrix will lead to excess accumulation of scar tissues.
OBJECTIVE: To investigate effects of bFGF on normal skin wound healing and hypertrophic scar formation.
METHODS: Normal and hypertrophic scar fibroblasts from tissue biopsies from 5 patients who underwent plastic surgery for repairing hypertrophic scars were isolated and cultured. The expressions of collagen, fibronectin and protein synthesis were detected by RT-PCR and ELISA. The mitochondrial membrane potential changes were measured using JC-1 staining and flow cytometry. Simultaneously, adenosine triphosphate (ATP) levels were determined by chemiluminescence method. The effects of bFGF on these indexes of normal and hypertrophic scar fibroblasts were observed.
RESULTS AND CONCLUSION: Hypertrophic scar fibroblasts become slower after being exposed to bFGF, which selectively inhibited type Ⅰ collagen production in hypertrophic scar fibroblasts ($P < 0.05$). Although bFGF inhibited type Ⅰ collagen production, it had no effect on type Ⅲ collagen expression in both normal and hypertrophic scar fibroblasts. However, fibronectin expression in the normal fibroblasts was up-regulated after bFGF treatment ($P < 0.05$). In addition, the mitochondrial membrane potential tended to depolarization, although no statistical difference, in hypertrophic scar fibroblasts treated with bFGF (10 or 100 μg/L). bFGF treatment increased the cellular ATP levels in the normal fibroblasts, while there were no significant alterations in the hypertrophic scar fibroblasts over a treatment of bFGF (10 or 100 μg/L, $P < 0.05$). The results suggest that there are differential effects and mechanisms on the skin fibroblasts with bFGF treatment in normal wound healing and hypertrophic scar formation.

INTRODUCTION

Wound healing is a dynamic and closely interactive process involving various growth factors, fibroblasts, and the formation of new blood vessels and extracellular matrices. 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, fibronectin and matrixase[1]. Nevertheless, dysregulation of this process, such as the abnormal coordination of cell proliferation, extracellular matrix and neovascularization formation, or remodeling of the wound matrix will lead to excess accumulation of scar tissues. Dysregulation can also lead to fibrotic disorders, such as keloid formation, morphea, and scleroderma[2].

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 Keloids and hypertrophic scars[3].

The aberrant fibroblast phenotype also seems to contribute to the hypertrophic scars or keloid, which may be due to the differential response of normal and hypertrophic scar 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 hypothesized that, during wound healing, the action of the hpearan sulfate-degrading enzymes activates bFGF in mediating the formation of new blood vessels, a process known as angiogenesis[6-7]. Indeed, previous *in vitro* and *in vivo* studies demonstrated that bFGF was critical in the control of scar formation and improved wound healing[8-9]. Postoperative administration of bFGF to patients resulted in the inhibition of hypertrophic scar formation, suggesting that bFGF may be a potential novel therapeutic tool in the treatment of hypertrophic scars in clinic[10], although, the underlying molecular mechanism of bFGF reducing the hypertrophic scar, but enhancing wound healing remains to be defined.

The present study investigated the role of bFGF during the regulation of type Ⅰ/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 and have either a positive or negative effect on the ability of fibroblasts to synthesize, deposit, remodel, and generally interact with the extracellular matrix. In addition, mitochondria may be an important mediator in extracellular matrix homeostasis and cell proliferation[11-13]. We, therefore, assessed the mitochondrial membrane potential and cellular adenosine triphosphate (ATP) production before and after bFGF treatment in these fibroblasts.

SUBJECTS AND METHODS

Design

An *in vitro* comparative cytology observation.

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Time and setting

The experiment was performed at Guangdong Key Laboratory of the Shock and Microcirculation Research, Southern Medical University, and Department of Burns, Guangdong General Hospital from March 2007 to January 2009.

Subjects

Fibroblasts were isolated from human skin biopsies that were obtained from five patients who underwent plastic surgery to correct excess scar tissue, including 4 males and 1 female, aged 14–35 years, with sustained deep partial- to full-thickness thermal burns.

Inclusion criteria

①Patients aged equal to or smaller than 35 years. ②Patients with sustained deep partial- to full-thickness thermal burns. ③Without any known systemic conditions. ④The informed consent was obtained from each patient.

According to the *Administrative Regulations on Medical Institution*, formulated by the State Council of the People's Republic of China[14], informed consents were obtained from themselves. In each case, the histologically normal skin was also taken to obtain normal fibroblasts.

Reagents and instruments are listed as follows:

Reagent and instrument	Source
Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	Sigma, St. Louis, MO, USA
Recombinant human bFGF	Invitrogen, Carlsbad, CA, USA
Monoclonal rabbit antibody against human type Ⅲ collagen, SABC-Cy3 rabbit IgG kit	Boster Corp, Wuhan, China
ELISA kit	Adlitteram Diagnostic Laboratories Inc., Shanghai, China
RNA isolation and RT-PCR kits	Bio-Rad Laboratories, Hercules, CA, USA
E.Z.N.A. total RNA kit	Omega Bio-tek, USA
PrimeScript™ RT-PCR kit	TaKaRa, Japan
Sircol Collagen Assay Kit	Biocolor, Newtownabbey, UK
CellTiter-Glo assay kit	Promega, Madison, WI, USA
5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)	Molecular Probes, Eugene, OR, USA
Automatic microplate reader (SpectraMax M5)	Molecular Devices, USA
FACScan instrument	Becton Dickinson, UK

Methods

Cell cultures and treatments

The fibroblasts were isolated from the dermis of the biopsies following digestion with 0.75% collagenase, and cultured in DMEM supplemented with 1% fetal bovine serum according to a method previously described with some modifications[15-16]. For our experiments, the fibroblasts in passage 2–15 were plated at a density of 5 ×10^9 cells/L in culture plates and were serum deprived using serum-free medium for 24 hours. The cells, at 70%–80% confluence, were treated with 0.1, 1.0, 10, 100 and 1 000 μg/L bFGF in a serum-free medium.

MTT assay

The fibroblasts were grown and treated with bFGF for 72 hours. The viabilities of normal human and hypertropic scar fibroblasts were quantitatively assessed using MTT (5 g/L). The culture plates were read using an automatic microplate reader preset at a wavelength of 550 nm. The changed cell viability was calculated by using the formula: (Atreated cells/A non-treated cells) ×100%.

Enzyme-linked immunosorbent assay (ELISA)

Type Ⅰ and Ⅲ collagen and fibronectin levels in the conditioned culture media of human skin fibroblasts cultures were measured using a commercially available ELISA kit. The levels of type Ⅰ and Ⅲ collagen and fibronectin were assayed in triplicate and measured using an automatic microplate reader 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 hours, and the total RNA from the cells was extracted and converted into cDNA synthesis using an E.Z.N.A. total RNA kit. The fibroblasts were further subjected to RT-PCR amplification of gene expression. A PrimeScriptTM RT-PCR kit was used in accordance with the manufacturer's instructions[17]. The following oligonucleotide primer sets were designed and synthesized:

Type Ⅰ collagen
Forward: 5'-CAT GCC GTG ACT TGA GAC TCA-3'
Reverse: 5'-AGG CGC ATG AAG GCA AGT T-3'
Product size: 292 bp

Type Ⅲ collagen
Forward: 5'-CGA GCT TCC CAG AAC ATC ACA-3'
Reverse: 5'-TTC GTG CAA CCA TCC TCC A-3'
Product size: 170 bp

Fibronectin
Forward: 5'-TTC CTT GCT GGT ATC ATG GCA-3'
Reverse: 5'-TAT TCG GTT CCC GGT TCC A-3'
Product size: 156 bp

β-actin
Forward: 5'-GCC CTG AGG CAC TCT TCC A-3'
Reverse: 5'-GCG GAT GTC CAC GTC ACA-3'
Product size: 101 bp

In particular, the reaction mixtures were denatured at

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95 ℃for 10 minutes, and then amplified for 30 cycles at 94 ℃ for 30 seconds, 55 ℃ for 30 seconds, and 72 ℃ for 1 minute with a final extension at 72 ℃ for 10 minutes and later stored at 4 ℃.After that, the PCR products were electrophoresed in a 2% agarose gel. The expression levels of type Ⅰ collagen, type Ⅲ collagen, and fibronectin were quantified and normalized with β-actin using densitometry.

Assaying 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 red fluorescence, but then assumes a monomer, emitting a green fluorescence when ΔΨm depolarizes. The fibroblasts were grown and treated with bFGF for 72 hours. Trypsinized and 10^5 fibroblasts were collected by centrifugation (10 minutes, 1 000 r/min). After being washed twice with PBS, the cells were incubated with 2 μmol/L JC-1 at 37 ℃ for 15 minutes in a humidified incubator. JC-1 was excited at 488 nm, and the aggregate signal (red) was analyzed at 590 nm (FL2). As was the monomer signal (green), analyzed at 525 nm (FL1), on a FACScan instrument, using CellQuest software (Becton Dickinson). The ratio of red to green JC-1 mean fluorescence was calculated as a relative mitochondrial membrane potential according to a previous study[18].

Measurement of cellular ATP levels

Relative levels of cellular ATP were assayed using the CellTiter 96 assay kit from Promega according to the manufacturer's instructions[19]. Briefly, 5×10^3 cells were cultured in a 96-well plate for 72 hours. After that, the luminescent output was read on an automatic microplate reader once the cells were mixed with 0.1 mL of the CellTiter substrate for 10 minutes. The cellular ATP levels were presented as a percentage of luminescence at each drug concentration to the luminescence of non-treated, normal cells.

Main outcome measures

Effects of basic fibroblast growth factor on collagen, fibronectin expression, and mitochondria in normal skin and hypertrophic scar fibroblasts.

Design, enforcement and evaluation

The second and fifth authors designed this study, the first, second, and third authors performed experiments, and all five authors evaluated experimental results. Blind assessment was not employed.

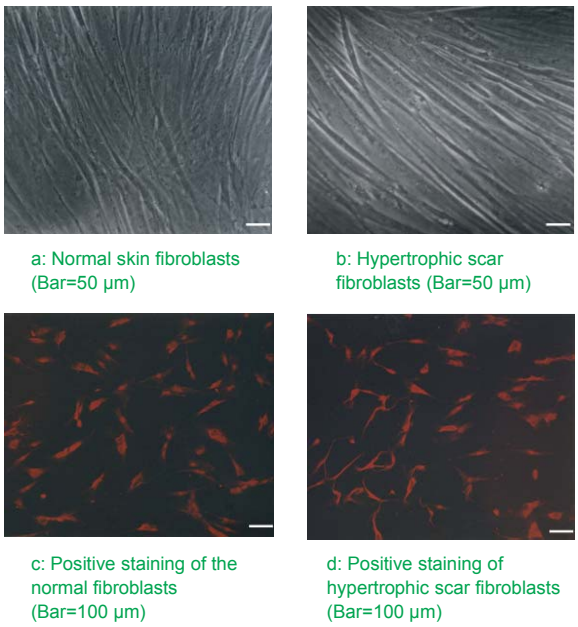
Statistical analysis

All data were presented as Mean ± SEM and analyzed with a Student's paired *t*-test and one-way ANOVA using SPSS 13.0 for Windows (SPSS, Chicage, IL, USA). A *P*-value equal to or less than 0.05 was considered statistically significant.

RESULTS

Isolation and identification of skin fibroblasts

As shown in Figure 1a, b, under a phase contrast microscope, the typical morphology for these fibroblasts, isolated from the normal human skin and hypertrophic scars were seen as an elongated spindle shape with clear cytoplasm. There were no morphological differences between two groups. These fibroblasts were then immunofluorescence-cytochemically stained with a type Ⅲ collagen antibody on monolayer cultures and tested positive for both types of fibroblasts (Figure 1c, d).

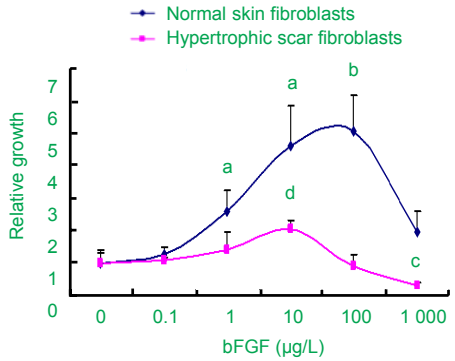


a: Normal skin fibroblasts (Bar=50 μm)
b: Hypertrophic scar fibroblasts (Bar=50 μm)
c: Positive staining of the normal fibroblasts (Bar=100 μm)
d: Positive staining of hypertrophic scar fibroblasts (Bar=100 μm)

Figure 1 Phase contrast and fluorescence photomicrographs of fibroblasts

Effect of bFGF on growth of these two types of fibroblasts form normal skin and hypertropic scars

The top level of growth in hypertrophic scar fibroblasts after bFGF treatment appeared 2 fold increase of the pre-treated value, which was much lower than 5 fold increase in normal fibroblasts (Figure 2). Moreover, the viability of these hypertrophic scar fibroblasts was significantly reduced to 30% after the addition of 1 000 μg/L of bFGF treatment ($P < 0.05$), which suggested that the hypertrophic scar fibroblasts had different response to bFGF compared to normal fibroblasts.

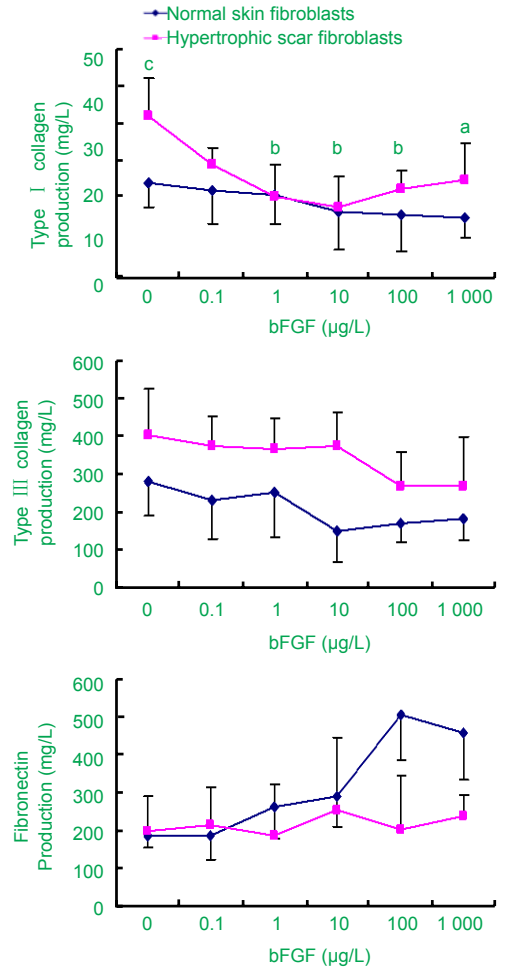


$^aP < 0.05$, $^bP < 0.01$ *vs*. untreated normal skin fibroblasts; $^cP < 0.05$, $^dP < 0.01$, *vs*. untreated hypertrophic scar fibroblasts

Figure 2 The dose-response curves of the normal skin and hypertrophic scar fibroblasts to basic fibroblast growth factor (bFGF)

Effect of bFGF in regulation of collagen expression in the conditioned medium

Next, collagen expression with or without bFGF treatment was detected. The type Ⅰ collagen in the hypertrophic scar fibroblasts was significantly higher than that in the normal fibroblasts in absence of bFGF ($P < 0.01$). However, the levels of type Ⅰ collagen in hypertrophic scar fibroblasts was decreased from (37.47±8.04) mg/L of pre-treated level to (26.65±3.79) mg/L after treatment with 0.1 μg/L of bFGF. In the presence of 1, 10, 100, and 1 000 μg/L of bFGF, the level of type Ⅰ collagen significantly decreased to (19.70±6.92) mg/L, (17.38±6.57) mg/L, (21.19±4.06) mg/L, and (23.42±7.79) mg/L, respectively ($P < 0.05$ or $P < 0.01$). In contrast, expression levels of type Ⅰ collagen in the normal fibroblasts were not significantly changed after treatment of different concentrations of bFGF when compared to the control fibroblasts (Figure 3). In addition, bFGF did not alter the production of the type Ⅲ collagen in both the normal and hypertrophic scar fibroblasts (Figure 3).

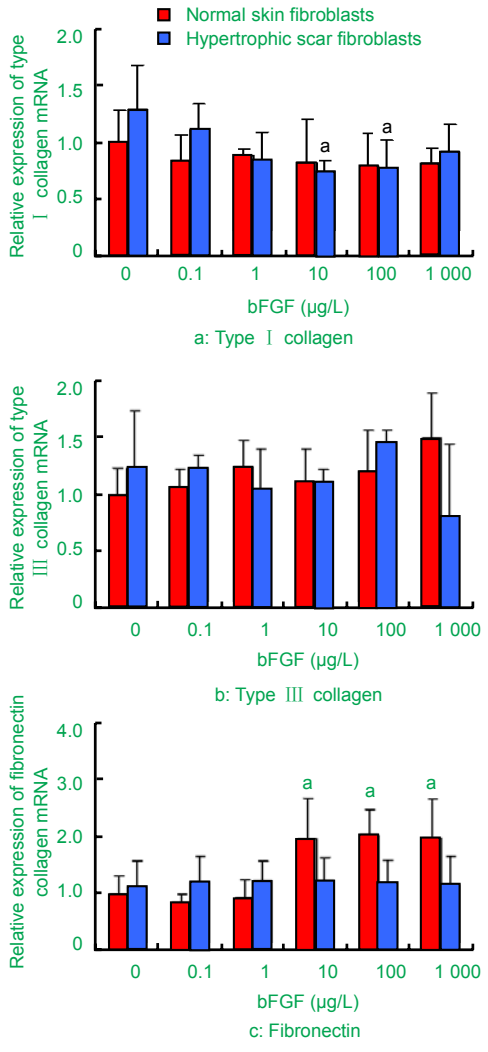


$^{a}P < 0.05$, $^{b}P < 0.01$, vs. 0 μg/L; $^{c}P < 0.01$, *vs.* normal skin fibroblasts

Figure 3 Effect of basic fibroblast growth factor (bFGF) on type Ⅰ/Ⅲ collagen and fibronectin expression in the normal and hypertrophic scar fibroblasts

Effect of bFGF in modulation of collagen mRNA expression

Expression levels of collagen mRNA in these fibroblasts was determined after bFGF treatment. Semi-quantitative RT-PCR analysis showed that after fibroblasts were treated with 10 and 100 μg/L of bFGF for 72 hours, the expression of type Ⅰ collagen mRNA was decreased by 25.2% and 23% in the normal fibroblasts respectively, while type Ⅰ collagen mRNA was induced even more in the hypertrophic scar fibroblasts after bFGF treatment ($P < 0.05$) (Figure 4). However, much higher concentrations of bFGF did not cause further inhibition of type Ⅰ collagen mRNA expression. The expression of the type Ⅲ collagen mRNA was unnoticeable after bFGF treatment (Figure 4).



$^{a}P < 0.05$, vs. 0 μg/L

Figure 4 Effect of basic fibroblast growth factor (bFGF) on the expression of type Ⅰ/Ⅲ procollagen and fibronectin mRNA in the normal and hypertrophic scar fibroblasts

Effect of bFGF on regulation of fibronectin expression

The bFGF effects on the expression of fibronectin mRNA and protein were assessed in both normal and hypertrophic scar fibroblasts. There was little difference in the expression of both the fibronectin protein and mRNA levels in the two types of fibroblasts in absence of bFGF. However, in the presence of bFGF, the normal fibroblasts showed an increase in the expression of fibronectin protein (Figure 3) and fibronectin mRNA (Figure 4), whereas the hypertrophic scar fibroblasts did not show any changes (Figures 3 and 4).

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

We treated these fibroblasts with 10 and 100 μg/L of bFGF. It was showed that no significant changes in the mitochondrial membrane potential were exhibited both in the normal fibroblasts and in the hypertrophic fibroblasts over a 72 hours treatment of bFGF (Figure 5). And bFGF treatment increased the cellular ATP levels in the normal fibroblasts, while there were no significant alterations of ATP level in the hypertrophic scar fibroblasts over a 72 hours treatment of bFGF (Figure 6).

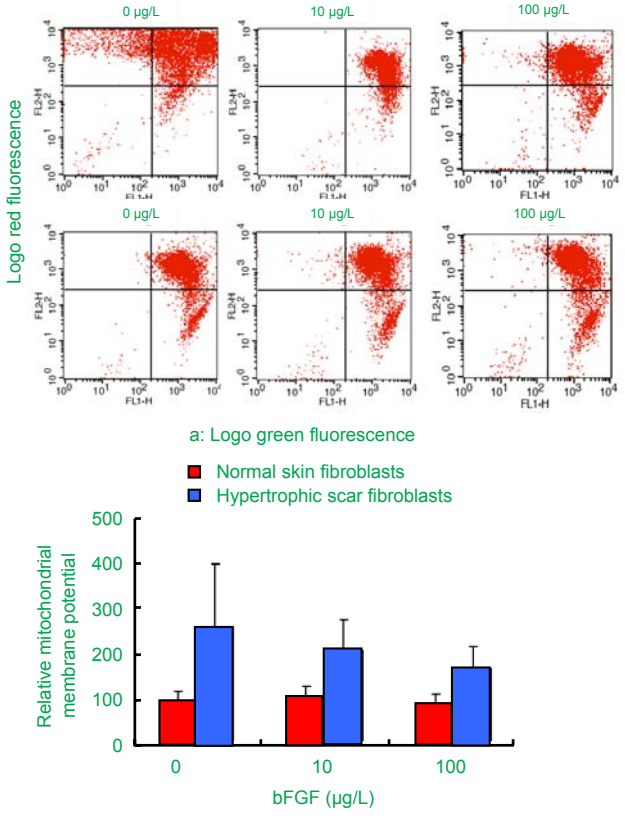


Figure 5 Flow cytometry analysis of mitochondrial membrane potential in the normal and hypertrophic scar fibroblasts

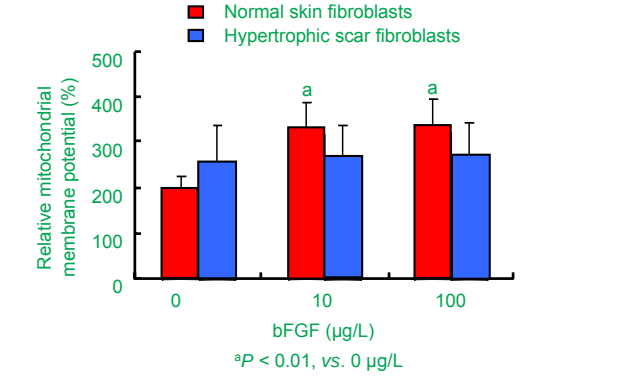


Figure 6 Effect of basic fibroblast growth factor (bFGF) on regulation of the cellular ATP levels in normal and hypertrophic scar fibroblasts

DISCUSSION

In the present study, we determined phenotypic alterations of the normal and hypertrophic scar fibroblasts after bFGF treatment. It was found that the growth of the hypertrophic scar fibroblasts increased slower than normal fibroblasts after exposure to bFGF. The hypertrophic scar fibroblasts were also more sensitive to bFGF-induced cell death. Furthermore, the hypertrophic scar fibroblasts produced more type Ⅰ collagen than that of the normal fibroblasts in absence of bFGF, whereas type Ⅰ collagen in hypertrophic scar fibroblasts was significantly inhibited by bFGF treatment in comparison to the normal fibroblasts. And bFGF increased fibronectin expression in the normal fibroblasts, but not so prominently in the hypertrophic scar fibroblasts. In addition, normal fibroblasts treated with bFGF (10 or 100 μg/L) showed no significant changes in the mitochondrial membrane potential. And the ATP level significantly increased in the normal fibroblasts after bFGF treatment. It is interesting to note that the mitochondrial membrane potential tended to depolarization, although no statistical difference, in hypertrophic scar fibroblasts treated with bFGF, which might explain why no changes of cellular ATP production in hypertrophic scar fibroblasts with bFGF treatment. The results indicated the different role of bFGF in the normal and hypertrophic scar fibroblasts for the regulation of wound healing. Future studies will be able to determine the different molecular mechanisms between the normal and hypertrophic scar fibroblasts.

As we know, excessive production and deposition of type Ⅰ collagen, the most abundant component of the extracellular matrix, plays an important role in hypertrophic scar formation[20]. A recent study showed that treatment with bFGF on scar tissue of a rat palate for six weeks dramatically suppressed formation of collagen type Ⅰ[21]. The inhibition occurred at the transcriptional level with down-regulation of the type Ⅰ collagen gene expression[8,22]. Our current data also support the inhibition and showed that bFGF significantly reduced the type Ⅰ collagen expression in the hypertrophic scar fibroblasts compared to that of the normal fibroblasts. Furthermore, it shows that type Ⅲ collagen synthesis is increased only in the early period of wound healing[23]. However, our current study showed this collagen might not be involved in bFGF-mediated wound healing. In addition, fibronectin participates in a provisional matrix and promotes fibroblast migration in the early phase of wound healing[24]. Excess deposition of fibronectin, at least in part, due to the regulatory mechanisms that function at the transcriptional levels, has been demonstrated in the formation of hypertrophic scars and keloids[25]. Our data demonstrated that bFGF increased fibronectin production in normal fibroblasts, but had less effect on the hypertrophic scar fibroblasts, suggesting that bFGF-mediated modulation of fibroblast growth, extracellular matrix production and gene expression is cell phenotype-dependent, although the underlying mechanism remains unclear.

Mitochondria are membrane-enclosed organelles found in most eukaryotic cells. They are usually termed to be the “power plants” in the cells. Mitochondria are also involved in a range of other cellular processes, such as cell signaling, differentiation, death, as well as the control of the cell cycle and cell growth.

During wound repair, a number of events become active, such as secretion of growth factors, cell migration, proliferation, and the production of the extracellular matrix, which are partly controlled by the energy production. It is evident that the extracellular matrix quantity and quality influence cellular growth, differentiation, morphology, survival, and mobility. Mitochondria can sense changes in extracellular matrix composition, and alterations in mitochondrial function also modify the extracellular matrix[26]. As a result, the different response of fibroblast mitochondria to bFGF between the normal and hypertrophic scar fibroblasts is important in determining bFGF regulating the scarless wound repair process. To date, such a study has not been documented in literature. In the present study, bFGF had no effect on the mitochondrial membrane potential in the normal skin fibroblasts, but tended to depolarized mitochondria in hypertrophic scar fibroblasts. Maintaining mitochondrial membrane potential was found to be a prominent mechanism in producing cellular ATP by means of the mitochondria[27-28]. Consistent with this mechanism, our results showed that bFGF, which induced an increment of relative ATP levels in the normal fibroblasts, had no effect on the cellular ATP in the hypertrophic scar fibroblasts. And growth of the normal fibroblasts increased faster than hypertrophic scar fibroblasts after exposure to bFGF. These findings suggest that the effect of bFGF on skin fibroblasts may occur with the involvement of the mitochondria. The proposal is of limited information that exists primarily as a potential and direct interaction of regulatory growth factor, such as bFGF, with the mitochondria during wound healing.

Taken together, our data demonstrated that *in vitro* exposure of fibroblasts to bFGF had different effects on fibroblast growth and extracellular matrix production between the normal and hypertrophic scar fibroblasts. Moreover, the effect of bFGF on these fibroblasts may be associated with the mitochondria for normal wound healing and hypertrophic scar formation. Thus, further investigation is needed to better understand the interaction of bFGF with the mitochondria during wound healing.

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碱性成纤维细胞生长因子对增生性瘢痕与正常皮肤成纤维细胞胶原、纤维连接蛋白表达的影响**☆

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摘要

背景：碱性成纤维细胞生长因子能促进愈合伤口产生胶原蛋白、纤维连接蛋白和基质酶的基质成分。然而，细胞增殖、细胞外基质及新生血管的形成或伤口基质重塑过程失调，会导致瘢痕组织过度增殖。

目的：观察碱性成纤维细胞生长因子在正常皮肤创面愈合和增生性瘢痕形成中的作用。

方法：从 5 例进行瘢痕修复手术患者身上同时取正常皮肤和增生性瘢痕组织，分离培养正常人皮肤成纤维细胞和增生性瘢痕成纤维细胞。应用 RT-PCR 和酶联免疫吸附法检测两种成纤维细胞胶原、纤维连接蛋白基因表达和蛋白合成。采用 JC-1 染色和流式细胞术测定成纤维细胞线粒体膜电位改变，采用化学发光法检测细胞内 ATP 水平改变。观察碱性成纤维细胞生长因子对两种细胞的上述指标的影响。

结果与结论：不同浓度碱性成纤维细胞生长因子可减慢增生性瘢痕成纤维细胞生长，抑制增生性瘢痕成纤维细胞Ⅰ型胶原表达和合成($P < 0.05$)。碱性成纤维细胞生长因子对正常皮肤和增生性瘢痕成纤维细胞Ⅲ型胶原表达和合成均无影响。然而可上调正常皮肤成纤维细胞表达纤维连接蛋白($P < 0.05$)。此外，10，100 μg/L 碱性成纤维细胞生长因子处理后增生性瘢痕成纤维细胞线粒体膜电位呈去极化趋势，正常皮肤成纤维细胞中 ATP 水平显著增高($P < 0.05$)。结果表明，碱性成纤维细胞生长因子在正常皮肤创面愈合和增生性瘢痕形成中可能有不同的作用和机制。

关键词：碱性成纤维细胞生长因子；增生性瘢痕；成纤维细胞；细胞外基质；线粒体；基因表达

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