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ADRENALINE INHIBITED CELL PROLIFERATION AND REGULATED EXPRESSION OF TGF-BETA1 AND BFGF IN CULTURED HUMAN HYPERTROPHIC SCAR FIBROBLASTS VIA α-RECEPTOR

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ABSTRACT

Adrenaline has been shown to modulate proliferation of mouse fibroblasts, adventitial fibroblasts and synovial B (fibroblasts-like) cells. However, little is known about the response of cultured human hypertrophic scar fibroblasts to adrenaline. In this study, we investigated cell proliferation and involved mechanisms in hypertrophic scar fibroblasts in response to adrenaline. Population doubling time (PDT) assay and MTT assay were performed to determine the cell proliferation and cell viability, respectively. The expression of bFGF and TGF-β1 was measured by reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA). The results showed that adrenaline inhibited proliferation of normal and hypertrophic scar fibroblasts in a dose-dependent manner. Moreover, adrenaline up-regulated the expression of bFGF and down-regulated the expression of TGF-β1 in normal and hypertrophic scar fibroblasts. Interestingly incubation with the α receptor antagonist regitine indicated that adrenaline mediated inhibition of cell proliferation and regulation of TGF-β1 and bFGF in cultured normal and hypertrophic scar fibroblasts were mediated by the α receptor. These studies suggest that adrenaline inhibits proliferation and alters the expression of TGF-β1 and bFGF in human hypertrophic scar fibroblast involving an α receptor mediated pathway.

Keywords: fibroblast – adrenaline – bFGF - TGF-β1

INTRODUCTION

Wound healing is a normal reaction to skin injury. It fulfills the necessary task of sealing the open wound, thus returning function to the skin. Ideally wound healing should be indistinguishable from normal uninjured skin, while this occurs in fetal wound healing. Adult wound healing usually results hypertrophic scar formation. The hypertrophic scar is one of problems of reparative surgery, which not only gives great burden to medicine but also does huge harms to patients.

The mechanism of hypertrophic scar is not clear. Recent studies (Razzaque and Taguchi, 2003; Hunt et al. 2000) suggest that wound healing is a complex process involving interactions among a variety of different cell types. The normal wound repair process consists of three phases – inflammation, proliferation, and remodeling –

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that occur in a predictable series of cellular and biochemical events. Wound healing is affected by several factors, including local factors (growth factors, edema and ischemia, low oxygen tension, and infection), regional factors (arterial insufficiency, venous insufficiency, and neuropathy), systemic factors (inadequate perfusion and metabolic disease), and other miscellaneous factors, such as nutritional state, preexisting illnesses, exposure to radiation therapy, and smoking. Large of investigations (Souchelnytskyi et al., 1996) suggest that growth factors play a role in cell division, migration, differentiation, protein expression, enzyme production and have a potential ability to heal wounds by stimulating angiogenesis and cellular proliferation, affecting the production and the degradation of the extracellular matrix, and being chemotactic for inflammatory cells and fibroblasts. There are seven major families of growth factors: epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukins (ILs), and colony-stimulating factor (CSF). The events of early wound healing reflect a finely balanced environment leading to uncomplicated and rapid wound healing. Chronic wounds, for many reasons, are out of balance. There are two key growth factors in the process of wound healing, that is, basic fibroblast growth factor (bFGF) and transforming growth factor β1 (Komarcevic, 2000; Tuan and Nichter, 1998). Basic FGF causes a dose-dependent inhibition of hydroxyproline biosynthesis, which is an index of collagen production. On the other hand, TGF-β1 stimulates collagen, elastin, and fibronectin synthesis while inhibiting extracellular matrix degradation.

Fibroblasts have important effects on the hypertrophic scar formation, so many drugs especially a series of hormones inhibiting fibroblast proliferation and synthesis or releasing ECM have been applied in clinical. Recent investigations suggest that adrenaline or noradrenaline may inhibit the cultured neonatal mouse fibroblasts and adventitial fibroblasts proliferation (Saito et al., 1997; Zhang et al., 2002). Mishima et al. (2001) also found that epinephrine or norepinephrine dose- and time-dependently decrease the number of synovial B (fibroblast-like) cells. Other studies show that the cell morphology and proliferating activity of postburn scar-derived fibroblasts could wholy be inhibited by Tripterygium extract (LLZ) which is similar to adrenaline (Xie et al., 2002). Our previous study also shows that adrenaline can inhibit proliferation and induce cell apoptosis of hypertrophic scar fibroblasts (Zhang et al., 2005). Therefore, the present investigation was designed to delineate the mechanism of adrenaline inhibiting proliferation and inducing apoptosis of hypertrophic scar fibroblasts.

MATERIALS AND METHODS

Cell culture

Primary cell lines of normal and hypertrophic scar dermal fibroblasts were established from operating room specimens. The Human Subjects Committee at Nanhua University provided exemption for the laboratory to use these operative specimens, which otherwise would have been discarded. A standard explant method was used to establish the primary fibroblast cultures from the operative specimens. This explant method was performed using sterile technique under a laminar flow hood. After all of the subcutaneous tissue was removed with a scalpel blade, the gross specimens were minced into small fragments in a petri dish. The specimens underwent antimicrobial treatment with phosphate-buffered saline (PBS) solution with 5 % penicillin/streptomycin/amphotericin (PSA). Minced fragments measuring approximately 1 mm^3 were then placed into scored collagen-coated 75 cm^2 tissue culture flasks containing 5 ml of explant tissue culture media (40 % fetal calf serum in Dulbecco's modified eagle medium with 1 % PSA and 1 % l-glutamine). These flasks were then stored in a humidified incubator containing a 5 % CO_2 atmosphere at 37 °C. The ex-

plant-containing flasks were examined daily under light microscopy for outgrowth of fibroblasts from the tissue fragments. Until light microscopy demonstrated such fibroblast outgrowth, explant tissue culture media was changed daily. Once fibroblast outgrowth approached confluence, the tissue fragments were removed. The fibroblasts were then trypsinized with 0.05 % trypsin and subcultured into culture flasks containing 10 ml of primary culture media (10 % fetal calf serum in Dulbecco's modified eagle medium with 1 % PSA and 1 % l-glutamine). All flasks were stored in a humidified incubator containing a 5 % CO_2 atmosphere at 37 °C. Primary culture media was changed twice a week. Successive cultures were passed at confluence.

Experiments were performed using early passage cells - passage the 3rd–8th generations of normal and hypertrophic scar fibroblasts. At the time of experimentation, confluent fibroblasts were released from the culture flasks with 0.05 % trypsin. After visualization with light microscopy demonstrated cell release from the flask wall, trypsin soybean inhibitor was placed into each flask in a 1:1 ratio to inactivate the trypsin Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) was then added to each resulting cell suspension. The concentration of each cell suspension was determined using phase-contrast microscopy.

Cell treatment

Equal numbers of normal or hypertrophic scar fibroblasts were plated and cultured for 72 h in DMEM. Cells were washed twice with PBS and then incubated in DMEM containing buffer (control) or regitine (3×10^{-6} μM) for 1 h, then added variable concentrations of adrenaline (0, 0.05, 0.10, 0.20 μM) into the media for 24 h. The doses and times were determined in our previous study.

Population doubling time (PDT) assay for cell proliferation determination

Normal and hypertrophic scar fibroblasts grown in 60 mm Petri dish were treated as described previously, then washed with PBS and stained by Giemsa. The resulting of cell clones from each Petri dish was counted using phase-contrast microscopy.

PDT (h) = (tx1g2) /lgn
(t: culture time; n: clones)

MTT assay for fibroblasts – cell viability determination

Colorimetric MTT assay was performed to assess cell viability. Briefly, normal and hypertrophic scar fibroblasts grown in 96-well plates treated as described previously. Then 20 μl of MTT (5 mg/ml, Sigma) in PBS solution was added into each well at a final concentration of 5 mg/ml and then the plate was further incubated for 4 h. All remaining supernatants were removed and 150 μl of DMSO was added into each well and mixed thoroughly to dissolve the formed crystal formazan. After 15 min of incubation to ensure all crystals being dissolved, the light absorption was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Viability was expressed as a percentage of absorbance values in treated cells to that in control cells.

LDL activity assays

The leakage into the media of LDH, an indicator of cell injury, was detected with an assay kit (Jiancheng BioEngineering, Nanjing, China) according to the manufacturer's instructions. Briefly, normal and hypertrophic scar fibroblasts grown in 96-well plates treated as described previously. At the end of the incubation, 30 μl of cell medium was taken out for the activity analysis of extracellular LDH, which could catalyze the conversion of lactate to pyruvate, and then reacted with 2,4-dinitrophenylhydrazine to give the brownish red

color in basic solution. After reaction, each sample was detected and the absorbance was read at wavelength 440 nm and the results were expressed as U/L of cell culture supernatants.

RT-PCR for TGF-β1 and bFGF

Briefly, after experimental treatments, normal and hypertrophic scar fibroblasts were washed twice with cold PBS. Then, total RNA was isolated using TRIZOL (GIBCO BRL). mRNA was then isolated using a mRNA isolation kit (TaKaRa Biotechnology) and was reversely transcribed into first-strand cDNA. Gene expression was analyzed by PCR. Aliquots of the resulting cDNA (3 µl) were then subjected to PCR amplification in the presence of 0.2 µl AmpliTaq polymerase (Promega, 5 u/µl), 0.5 µl each primer (20 µM, primer sequence: human bFGF (28 cycles), S: 5'-GCT CTT AGC AGA CAT TGG AAG -3'; AS: 5'- GTG TGT GCT AAC CTT ACC T-3', 275 bp (Takizawa et al., 2001); human GAPDH (28 cycles), S: 5'- GAG ATG ATG ACC CTT TTG GC -3', AS: 5'- GTG AAG GTC GGA GTC AAC G -3', 365 bp; human TGF-β1 (28 cycles), S: 5'- GCC CTG GAC ACC AAC TAT TGC T -3', AS: 5'- AGG CTC CAA ATG TAG GGG CAG G -3', 184 bp (Sakai et al., 2001), 0.5 µl dNTP (10 mmol/L), 1.5 µl $MgCl_2$ (25 mmol/L) and 2.5 µl PCR buffer in a total volume of 25 µl. Amplification was done in DNA Thermal Cycler under the following conditions: denaturation at 94 °C for 30 s, annealing for 30 s (55 °C for bFGF and GAPDH, 60 °C for TGF-β1), and extension at 72 °C for 30 s, with final extension at 72 °C for 5 min. Six microliters of each PCR product were subjected to electrophoresis on agarose gel in Tris acetic acid-EDTA buffer and stained with ethidium bromide. The gels were photographed and then analyzed by image analysis system (GSD8000, UVP, England). For each cDNA sample, the densitometric units of the amplified cDNA fragments were counted for semiquantitatively evaluation by normalization with the GAPDH band.

ELISA for TGF-β1 and bFGF

TGF-β1 and bFGF levels from cell culture supernatants were measured by ELISA kit (bFGF ELISA kit, RayBiotech, Inc. TGF-β1 ELISA kit, Bender Medsystems). Normal and hypertrophic scar fibroblasts grown in 96-well plates were treated as described previously. Then 100 µl of each cell culture supernatant were added into appropriate wells. The well with plate holder was covered and incubated for 2.5 hours at room temperature. The solution was discarded and washed 4 times with 1x Wash Solution (200 µl each). 100 µl of 1x prepared biotinylated antibody were added into each well and incubated for 1 hour at room temperature. The solution was discarded and washed 4 times with 1x Wash Solution (200 µl each). 100 µl of prepared Streptavidin solution were added into each well and incubated for 45 minutes at room temperature. The solution was discarded and washed 5 times with 1x Wash Solution (200 µl each). 100 µl of TMB One-Step Substrate Reagent were added to each well and incubated for 30 minutes at room temperature in the dark. 50 µl of Stop Solution were added into each well and read at 450 nm immediately. Results are expressed as TGF-β1 or bFGF level in pg per ml of cell culture supernatants.

Statistical analysis

A paired student's t-test and one-way ANOVA was used for comparison of mean values. Results are from three independent experiments, each are expressed as mean ± SEM. Statistical significance was defined as $P < 0.05$.

RESULTS

Cell proliferation of human normal and hypertrophic scar fibroblasts treated by adrenaline

The human normal and hypertrophic scar fibroblasts cell lines used in this study each exhibited exponential growth. Cellular

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viability ranged from 92.8 % to 100 %, with the mean viability of each cell type control and treatment group ranging from 92.8 % to 97.2 %. All control and treatment groups showed an initial decline in cell population after modulation, followed by a recovery of exponential proliferation. Population doubling time (PDT) was calculated for control group and treatment group in order to compare the rates of cell proliferation. PDTs were derived from exponential best-fit curves generated from the portion of each growth curve demonstrating exponential growth. The normal and hypertrophic scar fibroblasts control group had a PDT of 105.0 and 98.6 hours, respectively.

In hypertrophic scar fibroblasts, the addition of 0.05, 0.10 and 0.20 µM adrenaline to the experimental conditions resulted in significant longer PDT, increasing PDT by 11.2 %, 19.7 % and 20.9 %, respectively ($P < 0.01$). In normal fibroblasts, the addition of 0.05, 0.10 or 0.20 µM adrenaline to the experimental conditions also resulted in significant longer PDT, increasing PDT to 117.5, 126.0 and 129.0 hours, respectively. Of note, however, is that normal dermal fibroblasts or hypertrophic scar dermal fibroblast treated with 0.05, 0.10 or 0.20 µM adrenaline and regitine had no statistically significant lower cell clones at 24 hours than untreated fibroblasts ($P > 0.05$) (Table 1).

Table 1: Effect of adrenaline and regitine on Population doubling times of fibroblasts

Fibro-blasts type	Adrenaline concentr. (µmol/L)	Regitine concentr. (µmol/L)	Population doubling time (hours)
NFB	0	0	105.0 ± 2.4
NFB	0	3×10^{-6}	104.5 ± 1.8
NFB	0.05	0	117.5 ± 3.3*
NFB	0.10	0	126.0 ± 2.2*
NFB	0.20	0	129.0 ± 3.0*
NFB	0.05	3×10^{-6}	99.5 ± 1.2
NFB	0.10	3×10^{-6}	100.8 ± 0.7
NFB	0.20	3×10^{-6}	109.7 ± 1.3
SFB	0	0	98.6 ± 3.2
SFB	0	3×10^{-6}	103.0 ± 2.3
SFB	0.05	0	110.5 ± 3.1*
SFB	0.10	0	118.3 ± 2.7*
SFB	0.20	0	119.2 ± 2.5*
SFB	0.05	3×10^{-6}	98.9 ± 3.8
SFB	0.10	3×10^{-6}	101.6 ± 1.5
SFB	0.20	3×10^{-6}	107.2 ± 1.3

Population doubling times in cultured cells challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 µmol/L) and (or) regitine ($3x10^{-6}$ µmol/L). The Population doubling times are the mean ± SEM of 3 independent experiments.

** $P < 0.01$ vs the control group. NFB, normal human fibroblasts; SFB, human hypertrophic scar fibroblasts*

MTT assay showed that when hypertrophic scar fibroblasts treated with 0.05, 0.10 or 0.20 µM adrenaline, the cell viability was significantly decreased to 60.0 %, 42.0 % and 13.0 %, respectively ($P < 0.01$). In normal fibroblasts, the addition of adrenaline resulted in significant decrease of cell viability. On the other hand, if regitine and 0.05, 0.10 µM adrenaline stimulated normal or hypertrophic scar fibroblasts simultaneously, the cell viability did not significantly decrease compared with the control group. However, if normal or hypertrophic scar fibroblasts were incubated with regitine and 0.20 µM adrenaline at the same time, the cell viability also decreased, which were 78.5 % and 83.5 % respectively ($P < 0.05$). But it was still higher than the group of 0.20 µM adrenaline treated fibroblasts (Table 2).

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Table 2: Effect of adrenaline and regitine on the cell viability of fibroblasts

Fibroblasts Type	Adrenaline concentr. (μmol/L)	Regitine concentr. (μmol/L)	OD490	Cell viability
NFB	0	0	0.306 ± 0.012	1.00
NFB	0.05	0	0.184 ± 0.004	0.60*
NFB	0.10	0	0.129 ± 0.000	0.42*
NFB	0.20	0	0.039 ± 0.011	0.13*
NFB	0	3×10^{-6}	0.299 ± 0.001	0.98
NFB	0.05	3×10^{-6}	0.359 ± 0.000	1.00
NFB	0.10	3×10^{-6}	0.197 ± 0.018	0.97
NFB	0.20	3×10^{-6}	0.240 ± 0.000	0.785**
SFB	0.05	0	0.181 ± 0.003	0.577*
SFB	0.10	0	0.114 ± 0.017	0.363*
SFB	0.20	0	0.042 ± 0.000	0.133*
SFB	0	3×10^{-6}	0.314 ± 0.016	1.00
SFB	0.05	3×10^{-6}	0.295 ± 0.020	0.94
SFB	0.10	3×10^{-6}	0.27 ± 0.015	0.87
SFB	0.20	3×10^{-6}	0.262 ± 0.000	0.835**

The cell viability in cultured cells challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L). The cell viability is the mean of 3 independent experiments.

** P < 0.01 vs the control group.*
*** P < 0.05 vs the control group. NFB, normal human fibroblasts; SFB, human hypertrophic scar fibroblasts.*

To investigate the cell toxicity of adrenaline or/and regitine, we determined LDH leakage of cell culture supernatants. The results suggested that adrenaline or/and regitine had no cell toxicity to fibroblasts (Table 3).

Table 3: Effect of adrenaline and regitine on LDH leakage in cell culture supernatants

Fibroblasts Type	Adrenaline concentr. (μmol/L)	Regitine concentr. (μmol/L)	LDH leakage
NFB	0	0	28.1 ± 0.23
NFB	0.20	0	27.0 ± 0.54
NFB	0.05	3×10^{-6}	26.8 ± 0.65
NFB	0.10	3×10^{-6}	28.2 ± 0.12
NFB	0.20	3×10^{-6}	29.1 ± 0.09
SFB	0	0	24.8 ± 0.33
SFB	0.20	0	26.3 ± 0.42
SFB	0.05	3×10^{-6}	23.5 ± 0.43
SFB	0.10	3×10^{-6}	23.9 ± 0.48
SFB	0.20	3×10^{-6}	22.8 ± 0.53

The LDH leakage in cell culture supernatants challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L). The LDH leakage is the mean ± SEM of 3 independent experiments. NFB, normal human fibroblasts; SFB, human hypertrophic scar fibroblasts.

Effect of adrenaline on TGF-β1 and bFGF expression

Altered expression of cell TGF-β1 and bFGF expression has been demonstrated to play an important role in the induction of apoptosis. Therefore, we evaluated the effects of adrenaline and its receptor antagonist on the expression of these growth factors in normal and hypertrophic scar fibroblasts by RT-PCR and ELISA. As illustrated, adrenaline significantly enhanced bFGF mRNA expression in normal and hypertrophic scar fibroblasts and its protein production in cultured supernatants (Figure 1, Table 4.). On the contrary, TGF-β1 expression was down-regulated in response to adrenaline (Figure 2, Table 5). However, in the presence of regitine, the increment of bFGF expression was inhibited (Figure 1, Table 4), and the adrenaline-induced down-regulation of TGF-β1 was inhibited by regitine either (Figure 2, Table 5).

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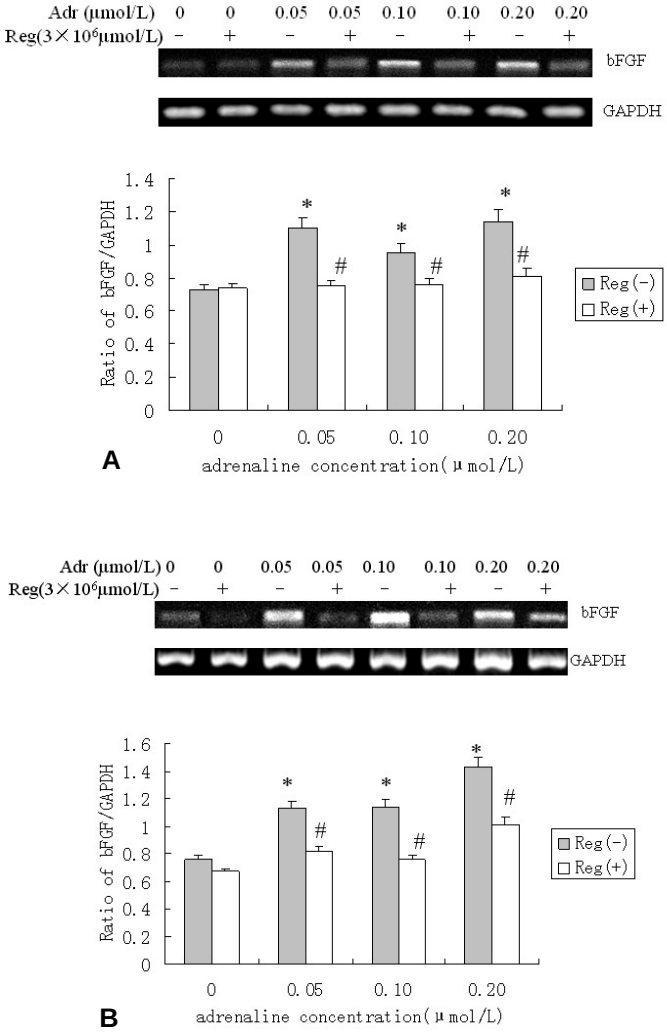

Figure 1: Kinetics of bFGF mRNA accumulation in cultured cells challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L), and samples were obtained for 24 hours. The expression of bFGF mRNA was determined by RT-PCR. GAPDH was used as an internal control. Blot shown is the representative of 3 experiments with similar results. Shown in the top panel is the representative of RT-PCR, and shown in the bottom panel are the mean ± SEM of 3 independent experiments.
* $P < 0.05$ *vs* the control group (adr, 0 μmol/L and Reg(-)). # $P < 0.05$ *vs* the Reg(+)group in the same adrenaline concentration group.
(A) Changes of bFGF mRNA expression in normal human fibroblasts after adrenaline and (or) regitine.
(B) Changes of bFGF mRNA expression in human hypertrophic scar fibroblasts after adrenaline and (or) regitine. NFB, normal human fibroblasts; SFB, human hypertrophic scar fibroblasts.

Table 4: Effect of adrenaline and regitine on the expression of bFGF in the cell culture supernatants

Fibroblasts Type	Adrenaline concentr. (μmol/L)	Regitine concentr. (μmol/L)	bFGF (pg/ml)
NFB	0	0	5.8 ± 0.14
NFB	0	$3×10^{-6}$	5.6 ± 0.23
NFB	0.05	0	10.9 ± 0.21*
NFB	0.10	0	11.1 ± 0.00*
NFB	0.20	0	18.6 ± 0.51*
NFB	0.05	$3×10^{-6}$	6.1 ± 0.17
NFB	0.10	$3×10^{-6}$	6.5 ± 0.41
NFB	0.20	$3×10^{-6}$	9.8 ± 0.21*
SFB	0	0	4.2 ± 0.38
SFB	0	$3×10^{-6}$	4.0 ± 0.31
SFB	0.05	0	15.3 ± 0.72*
SFB	0.10	0	19.1 ± 0.47*
SFB	0.20	0	28.1 ± 0.81*
SFB	0.05	$3×10^{-6}$	5.5 ± 0.16
SFB	0.10	$3×10^{-6}$	7.1 ± 0.25
SFB	0.20	$3×10^{-6}$	13.4 ± 0.53*

*Kinetics of bFGF protein in cultured cells supernatants challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L), and samples were obtained for 24 hours. The expression of bFGF protein was determined by ELISA. The results shown are the mean ± SEM of 3 independent experiments. * P < 0.01 vs the control group.*

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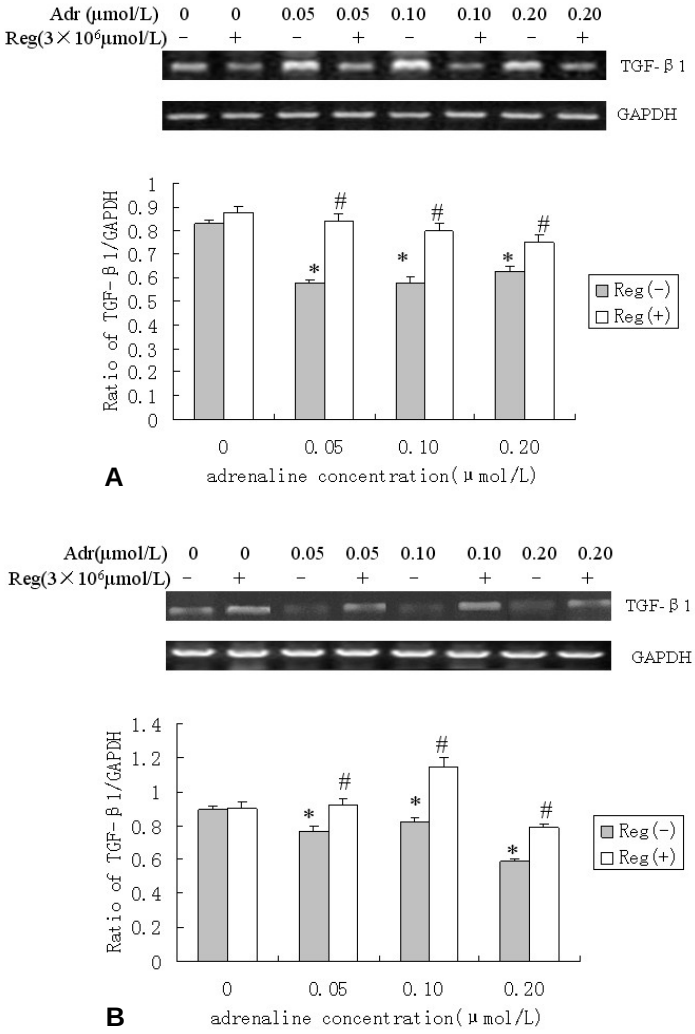

Figure 2: Kinetics of TGF-β1 mRNA accumulation in cultured cells challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L), and samples were obtained for 24 hours. The expression of TGF-β1 mRNA was determined by RT-PCR. GAPDH was used as an internal control. Blot shown is the representative of 3 experiments with similar results. Shown in the top panel is the representative of RT-PCR, and shown in the bottom panel are the mean ± SEM of 3 independent experiments.
* *P* < 0.05 *vs* the control group (adr, 0 μmol/L and Reg(-)). # *P* < 0.05 *vs* the Reg(+) group in the same adrenaline concentration group.
(A) Changes of TGF-β1 mRNA expression in normal human fibroblasts after adrenaline and (or) regitine.
(B) Changes of TGF-β1 mRNA expression in human hypertrophic scar fibroblasts after adrenaline and (or) regitine. NFB, normal human fibroblasts; SFB, human hypertrophic scar fibroblasts.

Table 5: Effect of adrenaline and regitine on the expression of TGF-β1 in the cell culture supernatants

Fibroblasts Type	Adrenaline concentr. (μmol/L)	Regitine concentr. (μmol/L)	TGF-β1 (pg/ml)
NFB	0	0	289.6 ± 8.77
NFB	0	$3×10^{-6}$	279.1 ± 5.91
NFB	0.05	0	199.7 ± 7.67*
NFB	0.10	0	146.7 ± 1.56*
NFB	0.20	0	130.1 ± 3.27*
NFB	0.05	$3×10^{-6}$	294.5 ± 5.91
NFB	0.10	$3×10^{-6}$	289.5 ± 5.27
NFB	0.20	$3×10^{-6}$	238.6 ± 2.28*
SFB	0	0	427.1 ± 5.63
SFB	0	$3×10^{-6}$	431.6 ± 4.73
SFB	0.05	0	381.1 ± 3.00*
SFB	0.10	0	337.4 ± 1.59*
SFB	0.20	0	364.0 ± 1.08*
SFB	0.05	$3×10^{-6}$	430.1 ± 2.96
SFB	0.10	$3×10^{-6}$	418.7 ± 5.62
SFB	0.20	$3×10^{-6}$	398.2 ± 2.34*

Kinetics of TGF-β1 protein in cultured cells supernatants challenged by adrenaline and (or) regitine. Cultured cells were challenged by adrenaline (0.05-0.20 μmol/L) and (or) regitine ($3x10^{-6}$ μmol/L), and samples were obtained for 24 hours. The expression of TGF-β1 protein was determined by ELISA. The results shown are the mean ± SEM of 3 independent experiments.
** P < 0.01 vs the control group.*

DISCUSSION

Damage inflicted by surgical or traumatic injury triggers wound healing, a necessary process that aims to quickly replace the injury with functional tissue. Intricate interactions between growth factors, proteases, and cells result in cellular proliferation with synthesis of the extracellular matrix. The hypertrophic scar is skin abnormalities that is unique to humans and is characterized by excessive deposition of collagen in the dermis and subcutaneous tissues secondary to traumatic or surgical injuries. A lot

of studies (Gauldie et al., 1999; Razzaque et al., 1999; Mutsaers et al., 1997; Razzaque and Taguchi, 2002) have shown that the proliferation of matrix-producing cells (fibroblasts) with subsequent overproduction and accumulation of matrix proteins contributes to various human and experimental fibrotic diseases. In the hypertrophic scar formation, there is the proliferation of dermal fibroblasts and overproduction of ECM, too. The hypertrophic scar presents major challenges in clinical practice because effective antifibrotic agents are not yet widely available.

The results from our study support that adrenaline alters cellular proliferation for human normal and hypertrophic scar dermal fibroblast populations in a dose-dependent manner. The normal and hypertrophic scar fibroblasts in our study responded to adrenaline, by slowing population doubling in a dose-dependent manner. Moreover, in normal and hypertrophic scar fibroblasts, the addition of 0.05, 0.10 and 0.20 μM adrenaline into the experimental conditions resulted in somewhat decrease of the cell viability, decreasing to 60 %, 42.0 %, 13.0 % and 57.7 %, 36.3 %, 13.3 %, respectively. Saito et al. (1997) also found that adrenaline, noradrenaline and cortisol among the circulation in postoperative patients inhibited the growth of cultured neonatal mouse fibroblasts. Our previous studies suggested that adrenaline also induced apotposis (Zhang et al., 2005).

To investigate the mechanism of adrenaline inducing apoptosis of fibroblasts, we used α receptor inhibitor - regitine addying into the experimental conditions. Modulation of normal or hypertrophic scar fibroblasts with regitine and adrenaline did not result in a statistically alteration in cell viability compared with controls. The results suggest that these responses to adrenaline in fibroblasts were inhibited by the alpha receptor inhibitor. Zhang et al. (2002) demonstrated that high norepinephrine concentrations (10 mM) inhibited adventitial fibroblasts proliferation and this inhibition was mediated by alpha (2)-ARs. Mishima et al. (2001) also found that epinephrine or norepinephrine dose- and time-dependently decreased the number of synovial B (fibroblast-like) cells.

Cytokines and growth factors secreted by inflammatory cells and by fibroblasts play an important role in the hypertrophic scar formation by regulating the proliferation of fibroblasts and the accumulation of ECM. Growth factors, such as bFGF and TGF-β1, may play a key role in the wounding healing. Recent studies (Takehara, 2000; Isaka et al., 2000; Liu et al., 2002; Polo et al., 1999) suggest that TGF-β1 is consistently found to be up-regulated in various human and experimental fibrotic diseases, and blocking its bioactivity can suppress the matrix production and modulate the fibrotic process. In addition, it dose not only increase the transcription of collagens, but also decreases its degradation by inhibiting collagenase activity through increase production of matrix metalloproteinase inhibitors such as TIMP and plasminogen activator inhibitor. Kamamoto et al. (2003) used an experimental model where collagen gels populated by human fibroblasts underwent progressive contraction, allowing the study of wound healing remodeling and found TGF-β resulting in an increase in gel contraction. A study (Luo et al., 2002) of SD rats found that in the groups receiving antisense ODN and recombinant plasmid, the expression of TGF-β1 mRNA and protein was reduced during 14 days after burn. In the control group, type I collagen mRNA occurred at the second week and reached a peak value at the fourth week, while the antisense groups kept low expression of type I collagen and showed mild inflammatory reaction and less synthesis of collagen. Then, they concluded that antisense TGF-β1 could prevent the scar formation during wound healing. Saed and Diamond (2003) also had similar results in human peritoneal fibroblasts. Now we have known that fetal skin wounds heal rapidly without the scarring and inflammation accompanying adult skin wounds. The mechanism of the scarless fetal repair is unclear. Series of studies led to the hypothesis that scarless fetal wounds

may be relatively TGF-β1 deficient. A development in vivo study of SD rats found that the level of TGF-β1 could not be detected in the early embryogeny, which is scarless wound healing, while was elevated after born (Hsu et al., 2001). The similar results also have been found in sheep and human (Beredjiklian et al., 2003; Chen et al., 2002). These findings suggest that anti-TGF-β1 therapeutic strategies may ameliorate scar formation in adult.

Basic fibroblast growth factor, a single-chain polypeptide of 146 amino acids, causes a dose-dependent inhibition of hydroxyproline biosynthesis, an index of collagen production and opposes the fibrotic signal of TGF-β1 by inhibiting collagen synthesis and stimulating collagen degradation (Tan et al., 1993). Carroll and Koch (2003) demonstrated that heparin decreased dermal fibroblast proliferation and collagen production by increasing the levels of basic fibroblast growth factor (bFGF). A recent study (Wan et al., 2002) suggested that bFGF could reduce the promoter activities of human alpha 1(I) procollagen gene and antagonize the role of TGF-β1 in up-regulating the promoter activities of human alpha 1(I) procollagen gene in normal skin and hyertrophic scar fibroblasts. Other studies (Funato et al., 1997; Akasaka et al., 2004; Spyrou and Naylor, 2002) showed that bFGF also promoted the apoptosis of hypertrophic scar fibroblast and inhibited fibroblast converting to myofibroblast.

The results of the present study demonstrate that adrenaline inhibits TGF-β1 protein synthesis but stimulates bFGF protein synthesis in human normal and hypertrophic scar fibroblasts. Moreover, alpha-receptor antagonist significantly alters the effects of adrenaline on these two growth factors. These results suggest that the adrenaline may inhibit fibroblast proliferation and induce fibroblast apoptosis by decreasing the expression of TGF-β and increasing the expression of bFGF.

In summary, adrenaline inhibits normal and hypertrophic scar fibroblasts proliferation by the binding to alpha-receptor. Adrenaline increases bFGF production and decreases TGF-β1 production by normal and hypertrophic scar fibroblasts probably through the pathway of alpha-receptor. Our study indicates that adrenaline may mediate its clinical effects on hypertrophic scar by altering cell proliferation and the production of bFGF and TGF-β1.

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