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Endostatin inhibits fibrosis by modulating the PDGFR/ERK signal pathway: an in vitro study*

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Abstract: Accumulating evidence indicates that endostatin inhibits fibrosis. However, the mechanism is yet to be clarified. The aim of this study is to evaluate the effect of endostatin on platelet-derived growth factor-BB (PDGF-BB)- or transforming growth factor β1 (TGF-β1)-induced fibrosis in cultured human skin fibroblasts, and to further examine the molecular mechanisms involved. Human dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) and serum-starved for 48 h before treatment. Cells were grouped as follows: "PDGF-BB", "PDGF-BB+ endostatin", "TGF-β1", "TGF-β1+endostatin", "endostatin", and "blank control". The fibroblasts were stimulated with either TGF-β1 or PDGF-BB for 72 h in order to set up the fibrosis model in vitro. The cells were co-cultured with either TGF-β1 or PDGF-BB and endostatin and were used to check the inhibiting effect of endostatin. A blank control group and an endostatin group were used as negative control groups. The biomarkers of fibrosis, including the expression of collagen I, hydroxyproline, and α-smooth muscle actin (α-SMA), were evaluated using an enzyme-linked immunosorbent assay (ELISA) and Western blot. The expression of phosphorylated PDGF receptor β (p-PDGFRβ), PDGFRβ, phosphorylated extracellular signal-regulated kinase (p-ERK), and ERK was detected using Western blot and immunofluorescent staining was used to explore the mechanisms. Both PDGF-BB and TGF-β1 significantly up-regulated the expression of collagen I, hydroxyproline, and α-SMA. Endostatin significantly attenuated both the PDGF-BB- and TGF-β1-induced over-expression of collagen I, hydroxyproline, and α-SMA. PDGF-BB and TGF-β1 both promoted the expression of PDGFR, ERK, and p-ERK. Endostatin inhibited the expression of PDGFR and p-ERK but did not affect the expression of total ERK. Endostatin inhibited hypertrophic scar by modulating the PDGFRβ/ERK pathway. Endostatin could be a promising multi-target drug in future fibrosis therapy.

Key words: Endostatin; Hypertrophic scar; Phosphorylated platelet-derived growth factor receptor (p-PDGFR); Extracellular signal-regulated kinase (ERK); Signal pathway

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1 Introduction

Cutaneous scarring is an inevitable result of deep skin injury. Hypertrophic scar is characterized by excessive collagen deposition and impaired functional or cosmetic outcomes. There are plenty of therapeutic interventions to treat hypertrophic scars, including operative and conservative treatments (Friedstat and Hultman, 2014); however, there are no effective ways to completely cure hypertrophic scar.

Endostatin is a strong endogenous angiogenesis inhibitor and commonly used as an anti-angiogenic therapy targeted against tumor vasculature. Recently, multiple other functions of endostatin have been discovered beyond its anti-angiogenic properties. For example, it can modulate androgen receptor function (Lee *et al.*, 2015) and prevent sepsis or acute kidney injury (Peng *et al.*, 2015; Mårtensson *et al.*, 2016).

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Accumulating evidence indicates that endostatin inhibits organ and skin fibrosis. Our previous study confirmed that systemic application of endostatin could prevent hypertrophic scar formation in the rabbit ear model (Ren *et al.*, 2013). Yamaguchi *et al.* (2012) found that endostatin-derived peptide ameliorates organ fibrosis that is triggered by transforming growth factor-β (TGF-β) and bleomycin in human and mouse tissues. However, the molecular mechanisms by which endostatin mediates fibrosis in fibroblasts remain poorly understood.

TGF-β is the primary factor that drives fibrosis (Meng *et al.*, 2016). Platelet-derived growth factor (PDGF) also plays a pivotal role in fibrosis (Andrae *et al.*, 2008). As a subtype of PDGF, PDGF-BB has a strong mitogenic effect on fibroblasts (Lin *et al.*, 2015). TGF-β and PDGF-BB have usually been employed as the fibrosis models in previous studies for their fibrosis-promoting effect (Cho *et al.*, 2016; Choi *et al.*, 2016). The PDGF receptor (PDGFR) signal pathway is involved in both angiogenesis and fibrosis and has become a new target for treating fibrosis. Interestingly, endostatin is also involved in these two important pathological processes. It was our hypothesis that endostatin has a close relationship with the PDGFR signal pathway.

In this study, we evaluated the effect of endostatin on fibrosis by using PDGF-BB- and TGF-β1-induced human skin fibroblast fibrosis models and further elucidated its underlying mechanisms.

2 Materials and methods

2.1 Reagents and antibodies

Primary antibodies against PDGFR-β, phosphorylated PDGF receptor β (p-PDGFRβ), α-smooth muscle actin (α-SMA), phosphorylated extracellular signal-regulated kinase (p-ERK), ERK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA) and recombinant endostatin (rE, Endostar™) was purchased from the Simcere Pharmaceutical Company (Nanjing, China). Both PDGF-BB and TGF-β1 were purchased from the Prospech Bio, Rehovot, Israel. The enzyme-linked immunosorbent assay (ELISA) kits of human hydroxyproline and collagen I were purchased from the Abbexa Company (Cambridge, UK) and the MTS kit from the Promega Company (Madison, WI, USA).

2.2 Culture of human skin fibroblasts

Human dermal fibroblasts were purchased from ATCC (Manassas, VA, USA) and cultured according to their recommendations. All experimental procedures were conducted in accordance with the relevant guidelines and regulations of the hospital's central labs. Cell passages 3–5 were used in this experiment and were cultured in Dulbecco's modified Eagle's medium (DMEM), then were seeded in 12-well plates at a density of 3×10^3 cells/well for 24 h and were serum-starved for 48 h before treatment.

2.3 Treatments and groups

The cells were divided into 6 groups: (1) control group; (2) endostatin group; (3) PDGF group; (4) "PDGF+endostatin" group; (5) TGF-β1 group; (6) "TGF-β1+endostatin" group. In the control group, fibroblasts were treated with serum-free DMEM for 72 h. In the endostatin group, cells were treated with endostatin for 72 h. The fibroblasts were treated for 72 h with 200 ng/ml PDGF-BB in the PDGF-treated group and with 10 ng/ml TGF-β1 in the TGF-β1-treated group. In the "PDGF+endostatin" group, fibroblasts were treated with 200 ng/ml PDGF-BB for 72 h and 5 μg/ml endostatin was added at the 24th hour. Lastly, in the "TGF-β1+endostatin" group, fibroblasts were treated with 10 ng/ml TGF-β1 for 72 h and 5 μg/ml endostatin was added at the 24th hour. All cells were cultured in serum-free DMEM.

2.4 MTT cytotoxicity assay

The toxicity of endostatin on fibroblasts was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human fibroblasts (3×10^3) were grown in 96-well plates in a medium containing 10% fetal bovine serum for 24 h. The fibroblasts were washed three times with serum-free DMEM and were serum-starved for 24 h to make cells quiescent. Subsequently, endostatin (0, 1.25, 2.5, 5, and 10 μg/ml in DMEM) was added to each well for either 24, 48, or 72 h. The MTT solution was added and then incubated at 37 °C for 3 h. The MTT solvent solution was added and incubated for 15 min; absorbance was recorded at 420 nm on an MR7000 microplate reader (Dynatech, NV, USA).

2.5 ELISA assay

The concentrations of collagen I and hydroxyproline proteins were determined using commercially available ELISA kits following the manufacturer's guidance and as described by Hu *et al.* (2012). Either a monoclonal antibody of collagen type I or hydroxyproline was added to the assay diluents (100 μl). Then 50 μl of either control or sample was added and incubated for two hours. Each well was washed three times with wash buffer. Two hundred microliters of either collagen type I or hydroxyproline conjugate was then added to each well for two hours. The wells were then washed further three times. The substrate solution (200 μl) was added for 30 min and the reaction was then stopped. The optical density was tested with a microplate reader. The concentrations of collagen I and hydroxyproline proteins were determined by comparing the optical densities with the standard curves. The supernatant fluid was harvested and measured three times.

2.6 Western blot analysis

The expression of PDGFR, p-PDGFRβ, ERK, p-ERK, and α-SMA was detected using Western blot analysis according to the standard protocol as described by Okada *et al.* (2013). Cells were scraped into the lysis buffer before separating the target proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferring them onto a polyvinylidene fluoride (PVDF) membrane. Membranes were probed overnight at 4 °C with the following primary antibodies: PDGFR-β (1:1000 dilution), p-PDGFR-β (1:1000 dilution), ERK1/2 (1:500 dilution), and p-ERK1/2 (1:500 dilution). GAPDH (1:2000 dilution) was used as an internal control. The levels of target protein bands were analyzed with ImageJ software (v.1.60) normalized using GAPDH.

2.7 Immunofluorescent staining

Fibroblasts were treated with 4% (0.04 g/ml) paraformaldehyde solution followed by 0.2% (v/v) Triton X-100. Immunofluorescent staining with the antibody against PDGFRβ was performed according to the manufacturer's instruction as described by Hu *et al.* (2012). DAPI reagent (4',6-diamidino-2-phenylindole, MP Biomedicals, 1:7500) was used to stain the nucleus.

2.8 Statistical analysis

Each experiment was repeated three times and data were recorded and analyzed with Prism 6.0 software. A one-way analysis of variance (ANOVA) was the chosen statistical technique for testing the hypotheses and results with a P-value <0.05 considered statistically significant.

3 Results

3.1 Toxicity of endostatin with MTT cytotoxicity assay

The toxicity of endostatin was tested by exposing human fibroblasts, for durations of 24, 48, and 72 h, to gradient concentrations of endostatin (0, 1.25, 2.5, 5, and 10 μg/ml) before assessment by MTT assay. No significant reduction of absorbance was observed at 0–5 μg/ml of endostatin after 24–48 h. A concentration of 5 μg/ml was therefore chosen as the working concentration and 48 h as the acting time. The dose–response curves are shown in Fig. 1.

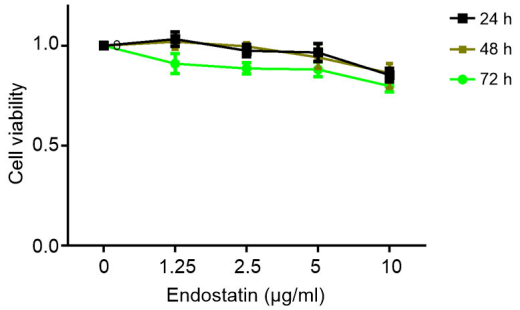

Fig. 1 MTT cytotoxicity assay (the dose–response curves) The toxicity of endostatin on fibroblasts was evaluated by MTT assay. No significant reduction in absorbance was observed at 0–5 μg/ml of endostatin after 24–72 h. We therefore chose 5 μg/ml as the working concentration and 48 h as the acting time

3.2 Effect of endostatin on fibrosis

Hydroxyproline was significantly decreased in the endostatin-treated groups compared with both the PDGF-BB and TGF-β1 groups (Fig. 2a) and the results were similar for collagen I (Fig. 2b). Endostatin significantly inhibited the overexpression of α-SMA in both the PDGF and TGF-β1 groups (Fig. 3).

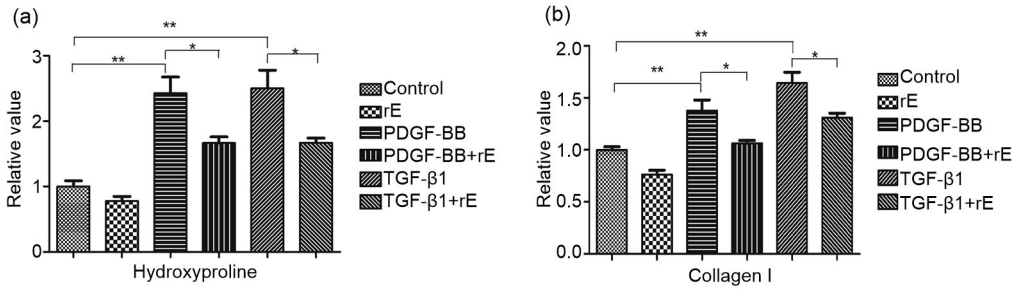

Fig. 2 Expression of collagen I and hydroxyproline with ELISA

Fibroblasts were grouped as follows: "PDGF-BB", "PDGF-BB+endostatin", "TGF-β1", "TGF-β1+endostatin", "endostatin", and "blank control" groups. The protein concentrations of collagen I and hydroxyproline proteins were determined using ELISA. (a) The hydroxyproline content in both PDGF-BB- and TGF-β1-induced groups was markedly higher than that in the control group (P<0.01), whereas the hydroxyproline content was significantly decreased in the endostatin-treated groups (P<0.01). (b) The results for the collagen I content were similar to the hydroxyproline results. For the statistics, data are expressed as mean±standard deviation (SD) (n=3). * P<0.05, ** P<0.01. rE: recombinant endostatin

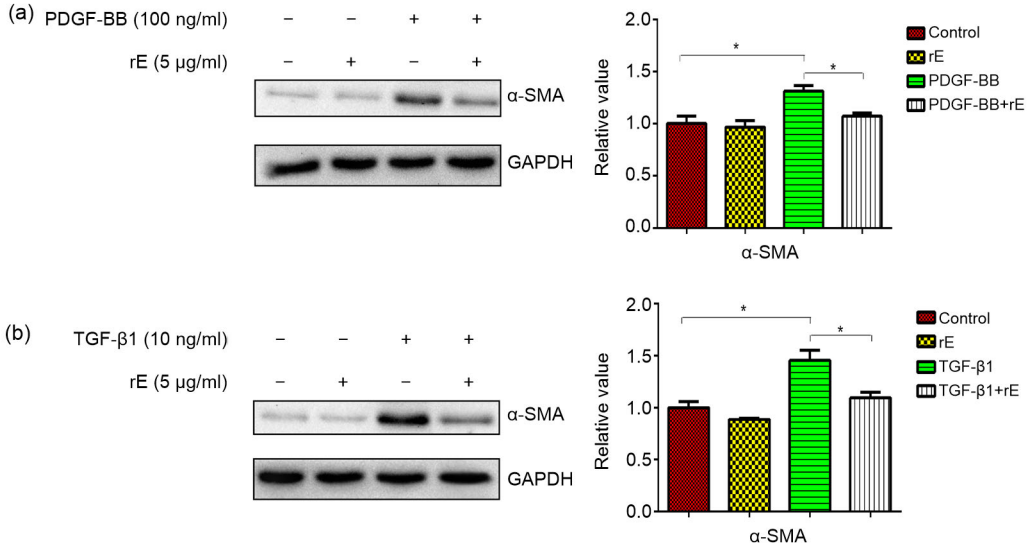

Fig. 3 Expression of SMA protein with Western blot analysis

Exposure of human fibroblasts to PDGF (a) or TGF-β1 (b) led to a significantly higher expression of the SMA protein. PDGF-BB- and TGF-β1-induced SMA expression was markedly inhibited by endostatin. Results were analyzed using ImageJ software (v.1.60) and the bands were quantified. For the statistics in this figure, data are expressed as mean±SD (n=3). * P<0.05. rE: recombinant endostatin

3.3 Inhibitive effect of endostatin on the expression of PDGFR, p-PDGFR, and p-ERK

Endostatin inhibited both the PDGF-BB- and TGF-β1-induced expression of PDGFR and p-PDGFR (Fig. 4). The expression of PDGFRβ was investigated using immunofluorescent staining (Fig. 5) and the result is consistent with that of Western blot. The p-ERK/ERK ratio was significantly increased in both PDGF-BB and TGF-β1 groups and decreased in the endostatin-treated group (Fig. 6).

4 Discussion

This study demonstrated that endostatin inhibits the protein expression of PDGFR and p-ERK, resulting in a reduction of scarring in a fibroblast fibrosis model.

Fibroblasts are activated by injury and other growth factors in the pathogenesis of fibrosis. Fibroblasts can differentiate into myofibroblasts to produce collagen (Bochaton-Piallat *et al.*, 2016). The productions of collagen and hydroxyproline are indicators

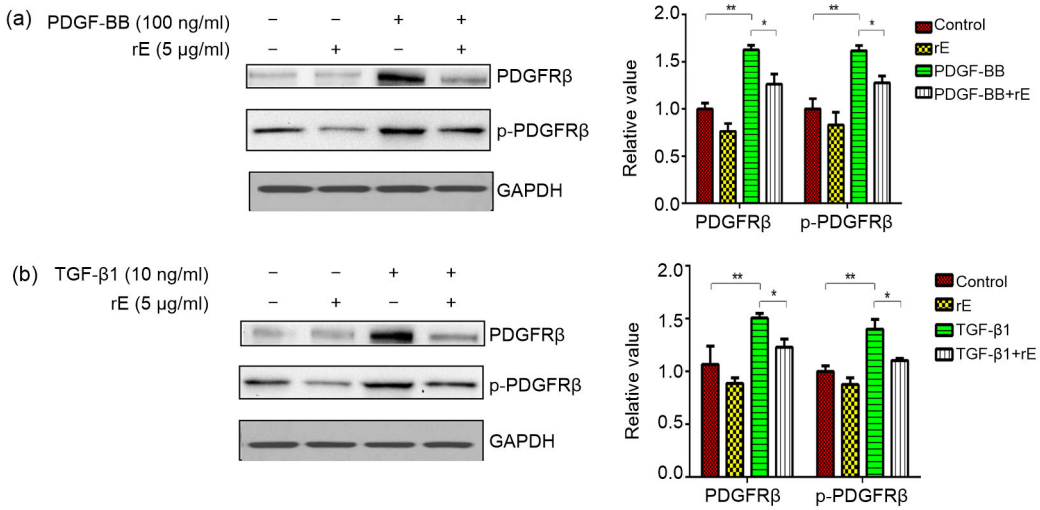

Fig. 4 Expression of PDGFRβ and p-PDGFRβ proteins with Western blot analysis

The expression of PDGFRβ and p-PDGFRβ was upregulated by both PDGF-BB (a) and TGF-β1 (b), but was significantly inhibited by endostatin. Results were analyzed using ImageJ software (v.1.60) and the bands were quantified. For the statistics in this figure, data are expressed as mean±SD (n=3). * P<0.05, ** P<0.01. rE: recombinant endostatin

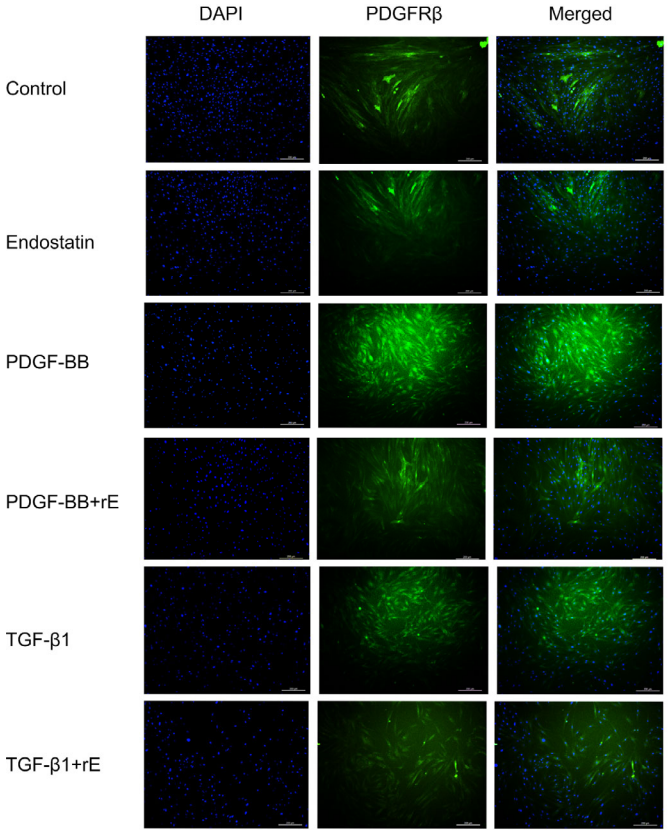

Fig. 5 Expression of PDGFRβ with immunofluorescent staining

Immunofluorescence using antibodies against PDGFRβ (green). DAPI was used to stain the nucleus (blue) in cultured human skin fibroblasts. The quantitation of the fluorescent images of PDGFRβ-positive cells was performed with the ImageJ software (v.1.60). The PDGFRβ-positive ratio in the PDGF-BB- and TGF-β1-induced groups is significantly higher than that in the blank control and endostatin groups (P<0.01). Endostatin markedly inhibited the expression of PDGFRβ compared with the PDGF-BB or TGF-β1 groups (P<0.05). Scale bar: 200 μm (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

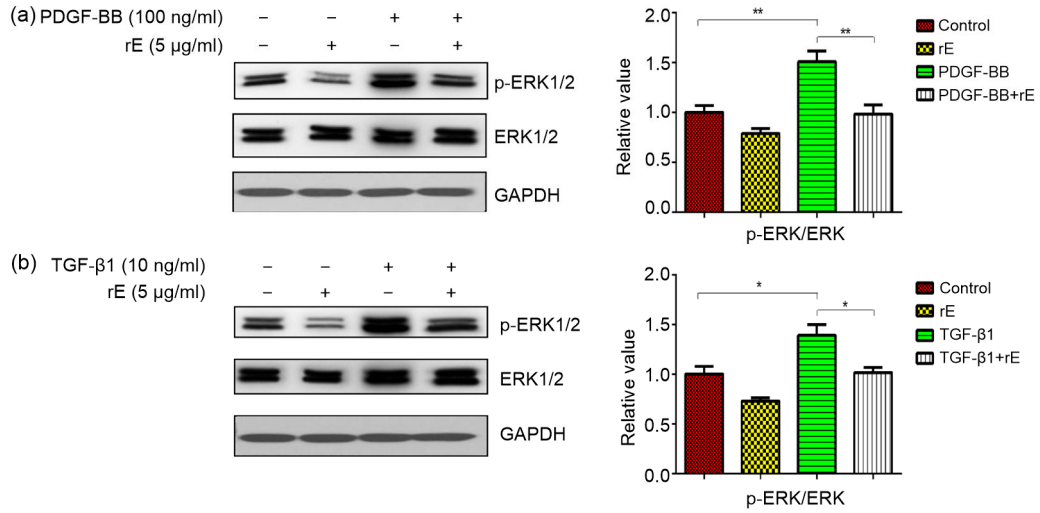

Fig. 6 Expression of ERK and p-ERK proteins with Western blot analysis

The p-ERK/ERK ratio was significantly upregulated by PDGF-BB (a) and TGF-β1 (b), but significantly decreased after treatment with endostatin. Results were analyzed using ImageJ software (v.1.60) and the bands were quantified. For the statistics in this figure, data are expressed as mean±SD (n=3). * P<0.05, ** P<0.01. rE: recombinant endostatin

for the degree of fibrosis (Tanaka *et al.*, 2010). Endostatin alleviated both PDGF-BB- and TGF-β1-induced overexpression of hydroxyproline and collagen I in human fibroblasts. α-SMA is a marker of myofibroblasts and plays a key role in wound healing and fibrosis (Bochaton-Piallat *et al.*, 2016). The results of the present study showed that the expression of α-SMA was increased by TGF-β1 and PDGF-BB and inhibited by endostatin. Endostatin was therefore thought to inhibit the transition from fibroblast to myofibroblast. To the best of our knowledge, this is the first study to investigate endostatin inhibition of human fibroblast fibrosis in vitro by modulating the PDGFR/ERK pathway.

The PDGFR signal pathway plays a critical role during epithelial-mesenchymal transition (EMT) in the progress of fibrosis and the overexpression of PDGF contributes to fibrotic disease (Tan *et al.*, 1995). Scar-derived fibroblasts express more PDGFR than those from normal skin (Haisa *et al.*, 1994). The PDGF/PDGFR signal pathway plays a pivotal role in angiogenesis and fibrosis (Tan *et al.*, 1995; Bonner, 2004; Wynn, 2008; Borkham-Kamphorst and Weiskirchen, 2015) by activation of transmembrane receptor tyrosine kinases (Heldin *et al.*, 2002; Mori *et al.*, 2008). The PDGF signaling pathway has become an effective target for inhibiting fibrosis (Fang *et al.*, 2013; Richeldi *et al.*, 2016).

The results of the present study showed that the expression of PDGFR was significantly increased in both TGF-β1- and PDGF-induced fibrosis. The activation of PDGFRβ resulted in increased collagen production. Endostatin inhibited the expression of PDGFR. The activation of the PDGF-BB/PDGFRβ-axis plays a role in fibrosis pathogenesis. Binding to PDGF could induce the dimerization and autophosphorylation of PDGFR (Jechlinger *et al.*, 2006), which could then transfer the signal by the downstream ERK cascade (Kaltalioglu and Coskuncevher, 2015).

ERK is in the downstream of the PDGFR signaling pathway and has a close relationship with cellular proliferation and migration (Reif *et al.*, 2003). PDGF-BB activates ERK by phosphorylation in mitogenesis (Tangkijvanich *et al.*, 2002). A high level of ERK phosphorylation has been demonstrated in scar-derived fibroblasts. In the present study, endostatin did not affect the expression of total ERK but inhibited the phosphorylation of ERK. These findings suggest that endostatin ameliorated dermal fibroblast fibrosis by suppressing the activation of ERK.

TGF-β1 and PDGF have close relationships with each other and both contribute to fibrotic disease (Tan *et al.*, 1995). PDGF promotes the expression of TGF-β receptor I/II and leads to a chain reaction of fibrosis (Tiede *et al.*, 2009). The expression of PDGF/PDGFR is significantly upregulated in TGF-β1-induced

EMT (Erawan *et al.*, 2008; Lehembre *et al.*, 2008). The results of the present study suggest that the expression of PDGFR is significantly increased in both TGF-β1- and PDGF-induced fibrosis, and decreases after treatment with endostatin.

There are some limitations to this study: (1) our study only investigated the mechanism of endostatin on fibrosis in vitro but not included the in vivo samples; (2) the downstream signal pathway of TGF-β1 was not investigated.

5 Conclusions

This study demonstrated that endostatin remarkably alleviated TGF-β1- and PDGF-BB-induced fibrosis in human fibroblasts by inhibiting the PDGFR/ERK pathway. Endostatin could be a promising multi-target drug in future fibrosis therapy.

Compliance with ethics guidelines

Yuan LI and Hai-tao REN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：内皮抑素通过调控 PDGFR/ERK 信号通路抑制人成纤维细胞纤维化的研究

目　的：近年来发现内皮抑素可以抑制组织纤维化，但是具体机制不详。本文旨在研究内皮抑素抑制纤维化的作用机制。

创新点：首次阐明了内皮抑素对成纤维细胞 PDGFRβ（血小板衍生生长因子受体 β）/ERK（细胞外调节蛋白激酶）信号通路的影响及其与纤维化的关系。

方　法：体外培养人成纤维细胞，采用血小板衍生生长因子-BB（PDGF-BB）或转化生长因子-β1（TGF-β1）建立细胞纤维化模型，进一步应用内皮抑素处理，通过检测胶原及肌成纤维细胞表面标志等了解内皮抑素对细胞纤维化和表型转化的抑制作用。进而检测内皮抑素对成纤维细胞 PDGFRβ/ERK 信号通路的影响，并分析其可能与抗纤维化作用存在的关联。

结　论：内皮抑素通过调控 PDGFRβ/ERK 信号通路抑制瘢痕增生，是多靶点的、具有较好的抗纤维化临床应用前景的药物。

关键词：内皮抑素；增生性瘢痕；血小板衍生生长因子受体；细胞外信号调节激酶；信号通路