Original Article

Effect of Avastin on the migration and invasion of pterygium fibroblasts

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Abstract

Purpose: To evaluate the effect of Avastin on human pterygium fibroblast migration and invasiveness.

Methods: VEGF secretion was compared between human pterygium fibroblasts and conjunctival fibroblasts by measuring VEGF-A by ELISA. The influence of Avastin on HPF migration and invasiveness was observed by wound scratch and Transwell migration assays. The expression of p-ERK1/2 and p-FAK was analyzed by western blotting.

Results: (1)VEGF was secreted in higher amounts by human pterygium fibroblasts than by conjunctival fibroblasts. (2) Avastin treatment decreased HPF migration and invasion. (3) Avastin significantly decreased the expression of p-ERK1/2 and p-FAK in human pterygium fibroblasts.

Conclusion: Avastin can inhibit migration and invasion of HPFs by decreasing the expression of p-ERK1/2 and p-FAK. (***Eye Science 2014; 29:214-218***)

Keywords: avastin (bevacizumab); pterygium fibroblast; migration; invasion; ERK1/2; FAK

Introduction

Pterygium is a common ocular surface proliferative disease that is exacerbated by exposure to ultraviolet light due to the activation of multiple cell signaling pathways, such as ERK (extracellular signal-regulated kinase), NF-κB (nuclear factor), and FAK (focal adhesion kinase) or to an imbalance of cytokines and growth factors[1]. Vascular endothelial growth factor (VEGF) is one of the most pivotal factors regulating angiogenesis and mitosis in vascular endothelial cells, which lead to the development of pterygium, and is closely correlated with tumor growth, invasion, and metastasis. Avastin is a monoclonal antibody of VEGF and has received U.S. FDA approval for use as a treatment for a variety of cancers. The application of Avastin in treating ocular surface and fundus ocular neovascular diseases is now being investigated.

At present, few studies have examined the use of Avastin in the management of pterygium and the mechanism underlying its effects remains elusive. The present study examines the influence of Avastin on the metastasis and invasion of human pterygium fibroblasts and provides support for the use of Avastin in preventing the growth and recurrence of pterygium.

Materials and methods

Study materials

Sample collection

A total of 30 patients diagnosed with primary pterygium and admitted to the Second Affiliated Hospital of Guangzhou Medical University between January and December 2012 were enrolled in this study.

Main instruments and reagents

The following materials and instrumentation were used: DMEM/F12 culture medium, streptomycin and penicillin antibody, fetal calf serum, trypsin, Avastin (bevacizumab), VEGF-A ELISA kits, Transwell chamber, p-ERK1/2 antibody, p-FAK antibody, microplate reader, electrophoresis equipment, and inverted phase difference microscope.

Methods

Cell culture

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The pterygium tissue was cut into 1 mm x 1 mm pieces and inoculated into a 6-well plate. The mixed cells that grew surrounding the pterygium tissue at 5-7 d were passaged at 80% cell confluence to obtain purified fibroblasts. Conjunctival fibroblasts were cultured by the same procedures.

Detection of VEGF in the cell supernatant

The cells were inoculated into a 6-well plate and the culture medium was replaced after a 72 h incubation. The culture medium was collected and stored in an Eppendorf tube at 4℃. The VEGF level secreted into the medium was determined following the manufacturer's instructions for the ELISA kit. The OD was measured at 450 nm using a microplate reader. The levels of VEGF were calculated based on a standard curve.

Detection of cell migration

Cultured cells at the logarithmic growth phase were inoculated into 6-well plates (2 ml in each well at a density of 2×10^5 cells/ml). When the cell density reached 70%-80%, a 200 μl pipette tip was used to make vertical scratch in the well and 2 ml serum-free culture medium and Avastin (2.5 g/L)were added. The cells were cultured at 37℃ in a 5% CO_2 incubator. The cells were divided into control (no Avastin) and experimental (Avastin; 2.5 g/l) groups. The width of the scratch was measured before (W0) and 24 h after (W24) incubation using an inverted phase difference microscope to determine cell migration. The wound closure rate (WC) was calculated from the equation: WC = (1-W24/W0) ×100%; The migration inhibition rate(MIR) was calculated based on the formula: MIR =(1-$WC_{Avastin}$/$WC_{control}$)×100%.

Detection of cell invasion

The polycarbonate membrane (diameter 8.0 μm) of a Transwell chamber was coated with Matrigel. Culture medium (500 μl containing 10% FBS) was added to the lower chamber of 24-well plate, which was then placed into the Transwell chamber. A serum-free cell suspension (100 μl, 2×10^5 cells/ml) was added to the upper chamber and cultured for 14 h. The cells were either control cells (no Avastin) or experimental cells (2.5 g/l Avastin). The Matrigel and cells in the chamber were removed with a cotton swab and the cells in the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet at room temperature. The cells were counted in 5 visual fields using a light microscope. The cells crossing the polycarbonate membrane were defined as invading cells (IC). The formula of IIR =(1-ICA_{vastin}/$IC_{control}$)×100% was adopted to calculate the IIR.

Expression of cell protein

The control (no Avastin) and experimental (2.5g/L Avastin) cells were lysed at 72 h after Avastin administration, the supernatant protein was collected, and the protein levels were determined by the BCA assay. A 25 μg sample of protein was loaded in each well for electrophoresis, followed by transfer onto a PVDF membrane (diameter 20 μm). The membrane was blocked with BSA for 30 min, incubated with primary antibody at 4℃ for 14 h, incubated with secondary antibody at room temperature for 1 h, and visualized with a developing agent.

Statistical analysis

SPSS 16.0 software was utilized for statistical analysis. The data were expressed as mean +/-s. Mean values between two groups were statistically compared by an independent sample *t*-test. $P<0.05$ was considered as statistically significant.

Results

Detection of VEGF secretion by pterygium and conjunctival fibroblasts

The amounts of VEGF secreted into the culture media of the control and Avastin groups, determined from standard curves, was 202.22±84.95 pg/ml and 78.13±54.075 pg/ml, respectively, and the difference was statistically significant ($P<0.05$).

Effect of Avastin on pterygium fibroblast migration

The WC rate in the control group was 80.5%±10.1%, which was significantly higher than the rate in the Avastin group(22.0%±11.2%, $P<0.01$). Avastin treatment significantly suppressed the migration of pterygium fibroblasts, which showed a MIR of 72.27%±14.7%. In the control group, the width of the scratch was significantly narrowed at 24 h, whereas the width of scratch in the Avastin group was only slightly reduced.

Effect of Avastin on pterygium fibroblast invasion

At 14 h after Avastin treatment, the quantity of ICs (20.0 ±4.2/visual field) was significantly decreased in the Avastin group compared with the con-

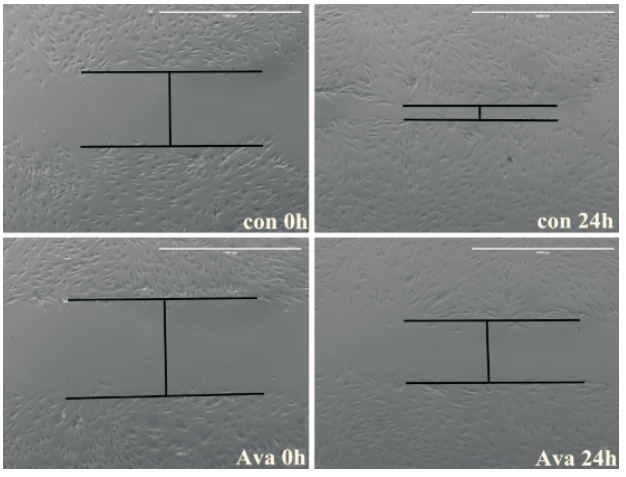

Figure 1 Comparison of the distance of cell migration between the control and Avastin groups at 0 h and 24 h after Avastin treatment.

trol group (34.6±5.1 per visual field). In the control group, the IIR was 43.21% ±9.46%(P=0.01) and the cells had an irregular shape and extended morphology, whereas the cells in the Avastin group had a regular spindle shape (Figure 2).

Table 1 Comparison of the quantity of invading pterygium fibroblasts in the control and Avastin-treated groups

Group	N	Cell count
Control group	5	34.6± 5.1
Avastin group	5	19.6± 4.0

T=5.139, v=8, P=0.01

Effect of Avastin on the expression of p-ERK1/2 and p-FAK proteins in pterygium fibroblasts

Figure 3 shows a significantly reduced expression of p-ERK1/2 and p-FAK proteins in the Avastin group compared with the control group (P<0.05).

Discussion

Pterygium is a highly invasive disease, characterized by abnormal fibroblast proliferation, neovascularization, collagen synthesis, and inflammatory cell infiltration[2]. Fibroblasts and VEGF both play pivotal roles in the development of pterygium. The pterygium fibroblasts investigated here had the ability to secrete VEGF and this secretion was significantly higher than in normal conjunctival fibroblasts, which is consistent with previous findings[3]. This finding suggests that paracrine and autocrine pathways probably exert a synergistic effect during the pathogenesis of pterygium. Blockage of the VEGF signaling pathway could inhibit the progression of ocular surface neovascular diseases from multiple aspects. The migration and invasion of pterygium fibroblasts are closely correlated.

Avastin has been proved to suppress the migration of retinal vessel endothelial cells[4]. In the present study, Avastin inhibited the migration of pterygium fibroblasts. The transwell assays confirmed the findings of the cell migration experiments. The quantity

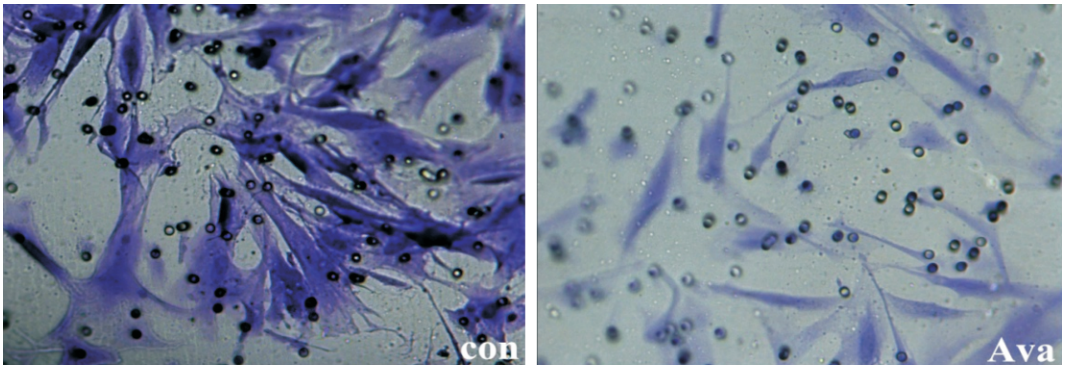

Figure 2 Comparison of the quantity of invading pterygium fibroblasts between the control and Avastin-treated groups

of cells penetrating the basement membrane and invading the lower chamber transwell was significantly decreased in the Avastin treated group compared with the control group. The transwell assay is the most common in vitro method for evaluating cell invasion. In the present study, the Transwell insert was equipped with a Matrigel-coated Millipore filter. The Matrigel used in this experiment is a soluble component of the basement membrane extracted from EHS (Engelbreth Holm Swarm) mouse sarcoma. Therefore, it can mimic in vitro the in vivo interaction between invading cells and the basement membrane.

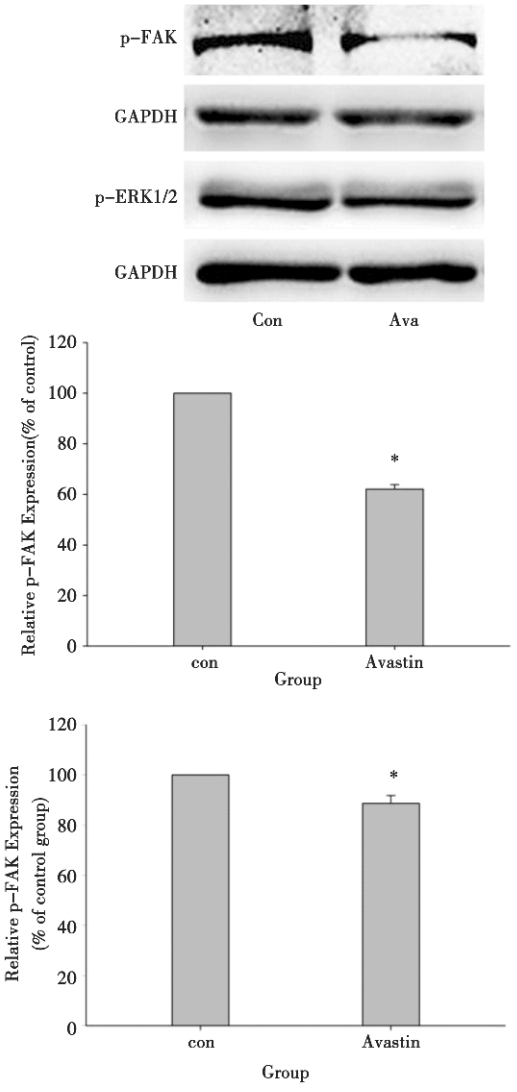

Figure 3 Expression of p-FAK and p-ERK1/2 proteins detected by Western blotting (* denotes $P<0.05$ compared with control group)

The present findings suggest that Avastin can inhibit the in vitro migration and invasion of pterygium fibroblasts by blocking the VEGF signaling pathway.

Avastin also suppressed the activation of ERK1/2 and FAK proteins, as determined by Western blotting. FAK, a type of non-receptor tyrosine kinase, is involved in a variety of signaling pathways and multiple biological processes, such as cell proliferation, apoptosis, and collagen metabolism. The activation of FAK may cause cascade reactions through the ERK signaling pathway that participate in cell migration and invasion, thereby leading to cell infiltration[5]. ERK consists of two isomers, ERK1 and ERK2 (p44and p42MAPKs or MAPK1 and MAPK2). Activated ERK participates in a wide range of physiological and pathological activities of cells[6].

Previous studies have demonstrated that VEGF can stimulate the synthesis of endothelial cell DNA mainly via activation of the PLC-γ-PKC-Raf-MEK-ERK signaling pathway by VEGFR-2, which activates DNA synthesis via intranuclear signal transduction and accelerates the proliferation of endothelial cells[7]. Wang and Zheng found that VEGF-A proliferation was mediated by the MEK1/2/ERK1/2 signaling pathway in human and bovine placental vascular endothelial cells[8,9]. Brain cells from wild type mice under hypoxic conditions showed a reduction in pERK1/2 expression following antibody neutralization of VEGF expression and blockage by siRNA[10], suggesting that FAK and ERK1/2 phosphorylation are the downstream reactions of the binding of VEGF and VEGFR-2.

The present study implicates binding of Avastin to the VEGFR-2 receptor, which then inhibits the activation of ERK1/2 and FAK proteins and suppresses cellular migration and invasion by blocking VEGF binding. However, the precise interaction and mechanism and the participation of different signaling pathways remain to be elucidated.

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