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A C-terminal fragment BIGH3 protein with an *RGDRGD* motif inhibits corneal neovascularization *in vitro* and *in vivo*

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ABSTRACT

An Arg–Gly–Asp (RGD) motif in the fourth FAS1 domain of the human BIGH3 (transforming growth factor-β1-inducible gene-h3) protein has been reported to play an important role in mediating tumor angiogenesis. The aim of this study was to investigate the inhibitory effect of a modified C-terminal fragment BIGH3 protein with an *RGDRGD* motif on corneal neovascularization *in vitro* and *in vivo*. Recombinant C-terminal fragment BIGH3 protein with wild-type sequence and modified C-terminal fragment BIGH3 protein containing an *RGDRGD* motif were successfully expressed and purified. We demonstrated that both proteins significantly inhibited vascular endothelial growth factor (VEGF)-induced human umbilical vein endothelial cell (HUVEC) adhesion, migration, and tube formation and induced cell apoptosis but failed to inhibit HUVEC proliferation. We determined that the mechanism underlying this activity was an interaction between BIGH3 and αvβ3 integrin, which blocked the phosphorylation of PI3K/Akt and ERK signaling pathways. The inhibitory effects of wild-type and modified C-terminal fragment BIGH3 proteins on angiogenesis were confirmed by a rabbit corneal neovascularization assay. More importantly, we provided evidence that the modified C-terminal fragment BIGH3 protein with an *RGDRGD* motif inhibited angiogenic activity far more effectively than did wild-type C-terminal fragment BIGH3. Collectively, our data show that a C-terminal fragment BIGH3 protein containing an *RGDRGD* motif might be promising as an effective drug in treating corneal neovascularization.

1. Introduction

Neovascularization plays important roles in several diseases of the eye, which results in vision impairment or blindness. However, much remains unknown about the pathogenesis of corneal neovascularization, especially at the molecular level. Neovascularization involves changes in vascular permeability, endothelial cell adhesion, migration, proliferation, and differentiation (Folkman and D'Amore, 1996). These processes depend on a number of growth factors as well as adhesion to the extracellular matrix (ECM) (Risau, 1997; Cheresh and Stupack, 2008; Bao et al., 2009). ECM-associated proteins, such as fibronectin (FN), vitronectin (VN), and BIGH3, are deposited into an adhesive fibrillar network and control cellular growth, differentiation, migration, and other functions by transmitting signals to the cells through specific integrins (Giancotti and Ruoslahti, 1999; Chavakis et al., 2004).

Studies of the molecular basis of neovascularization have identified a number of growth factor receptor pathways that promote neovascularization. Similarly, several angiogenesis inhibitors have been identified and applied as clinical treatments, including prolactin (Yoon et al., 2005; Nguyen et al., 2011), angiostatin (Nishioka et al., 2011), cyclosporin A (Nacev and Liu, 2011), TNP-470 (van Wijngaarden et al., 2010), steroids (McNatt et al., 1999), and endostatin (Pan et al., 2011).

BIGH3 (transforming growth factor-β1-inducible gene-h3) protein is an extracellular matrix protein whose expression is strongly induced by transforming growth factor (TGF)-β1 (Nam et al., 2003). BIGH3 cDNA sequence analysis revealed that the 683-amino acid BIGH3 protein contains four internal repeat domains (FAS1) and

Abbreviations: CNV, corneal neovascularization; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; RGD, Arg–Gly–Asp; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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two highly conserved sequences (H1 and H2) in each repeat. This protein also contains an *RGD* sequence, which may serve as an integrin recognition sequence. The fourth repeat of the FAS1 domain is specifically known to be involved in cell growth, differentiation, tumorigenesis, wound healing, and apoptosis (Kim et al., 2000a,b, 2003; Bae et al., 2002). Recombinant BIGH3 and the BIGH3 FAS1 domain alone significantly inhibited osteoblast differentiation *in vitro* (Thapa et al., 2005). The *RGD* sequence at the C-terminus of BIGH3 is located from amino acid positions 642–644, and an identical sequence is found in many extracellular matrix molecules that support cell adhesion, including fibronectin, vitronectin, laminin, collagen, osteopontin, and bone sialoprotein, all of which are recognized by some integrins (LeBaron et al., 1995). Furthermore, Nam et al. demonstrated that the *RGD* motif was a potential integrin recognition sequence and that it inhibited the adhesion and proliferation of endothelial cells (Nam et al., 2003). Murata reported that a poly(*RGD*) peptide was able to inhibit cell adhesion and proliferation better than a monovalent *RGD* peptide (Murata et al., 1991).

We previously reported that a modified endostatin containing an *RGDRGD* motif was a far more effective anti-angiogenesis factor than the wild-type endostatin gene (Ge et al., 2011). The purpose of the present study was to evaluate the anti-angiogenic effect of the C-terminal fragment BIGH3 protein with an *RGDRGD* motif on VEGF- induced proliferation, adhesion, migration, tube formation and apoptosis by HUVECs *in vitro*, and on corneal neovascularization *in vivo*. Furthermore, the integrin and signaling pathway related to its anti-angiogenic effect was examined.

2. Materials and methods

2.1. Cloning, expression, and purification of a modified C-terminal fragment BIGH3 protein with an RGDRGD motif

2.1.1. Site-directed mutagenesis

The plasmid pMD18-T/BIGH3 was obtained as previously described (Ge et al., 2006, 2008). The following primers were used to amplify the C-terminal fragment BIGH3, encoding amino acids 502 to 683 including amino acids 502 to 632 of the fourth FAS1 domain (Kim et al., 2000a,b), and to modify the C-terminal fragment BIGH3 gene by PCR and rapid site-directed mutagenesis, respectively: C-terminal fragment BIGH3, 5′-GGAGATATGGAGGTCTGATGTGTC-3′ (sense) and 5′- GACGATCTAATGCTTCATCCTCTCTAATAAC-3′ (antisense); modified C-terminal fragment BIGH3 with *RGDRGD* motif, 5′-GAGGGGATAGAGGAGACTCTGCGCTTG-3′ (sense) and 5′-GCGCAGAGTCTCCTCTATCCCCTC-3′ (antisense) (Shanghai Yingjun Biological Engineering Technology, Shanghai, China). The PCR reaction was performed in 35 cycles of 5 min at 95 °C, 60 s at 94 °C, 90 s at 56 °C, and 2 min at 72 °C, followed by 10 min at 72 °C. The site-directed mutagenesis PCR reaction was carried out in 15 cycles of 5 min at 95 °C, 60 s at 94 °C, 60 s at 55 °C, and 5 min at 72 °C, followed by 10 min at 72 °C. The products of site-directed mutagenesis PCR were isolated by ethanol precipitation and digested by *Dpn*I (New England Biolabs, USA). The digestion product was transformed into *Escherichia coli*, and positive clones were verified.

2.1.2. Construction of expression plasmids

The wild-type and modified C-terminal fragment BIGH3 genes were subcloned into the *Xba*I-*Sal*I sites of the pET32a^{+} vector, which was then introduced into *E. coli* DH5α (Invitrogen, USA) and selected with ampicillin. After the recombinant plasmids were verified by *Xba*I and *SalI* (New England Biolabs, USA) restriction digest, they were introduced into *E. coli* BL21 (DE3) (Novagen, USA) and selected with ampicillin. The sequences were further confirmed by automated sequencing (Shanghai Sangon Biological Engineering Technology, Shanghai, China). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

2.1.3. Expression and purification of recombinant C-terminal fragment BIGH3 proteins

The expression and purification of recombinant C-terminal fragment BIGH3 proteins (wild-type and *RGDRGD* mutated forms) was carried out as previously described (Yuan et al., 2004). Briefly, an overnight culture of BL21 (DE3) harboring pET32a/C-terminal fragment BIGH3 or pET32a/*RGDRGD*-C-terminally fragment BIGH3 was diluted 1:100 in LB and incubated at 37 °C until an OD_{600} of 0.6 was reached. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After growing at 37 °C for 4 h with gentle shaking, *E. coli* were harvested by centrifugation, and the pellets were resuspended in BugBuster (Novagen, USA) solution with benzonase, according to the manufacturer's specifications. After incubation at room temperature for 20 min with constant rocking, inclusion bodies were separated from the soluble fraction by centrifugation at 10,000 × *g* for 30 min at 4 °C. The inclusion bodies were resuspended in 50 ml of a solution containing 6 M guanidine hydrochloride, 100 mM potassium phosphate, and 10 mM Tris–HCl pH 8.0. This suspension was then shaken overnight to solubilize the protein. After centrifugation, the supernatant was collected and loaded onto a pre-equilibrated 5 ml Ni-NTA Superflow agarose column (Qiagen, USA). The protein-bound Ni-NTA resin was first washed with 50 ml of a buffer containing 8 M urea, 100 mM potassium phosphate, and 10 mM Tris–HCl pH 8.0, followed by a wash with 50 ml of a buffer containing 30 mM imidazole, 8 M urea, 100 mM potassium phosphate, and 10 mM Tris–HCl pH 8.0. The bound recombinant C-terminal fragment BIGH3 proteins were then eluted in a buffer containing 150 mM imidazole, 8 M urea, 100 mM potassium phosphate, and 10 mM Tris–HCl pH 7.4.

2.1.4. Refolding experiments

To obtain bioactive recombinant C-terminal fragment BIGH3, protein refolding was performed with a FoldIt screening kit (Hampton Research, CA) according to the manufacturer's specifications. The purified wild-type and modified C-terminal fragment BIGH3 proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma, USA) and Western blot. The primary antibody used was a full-length mouse polyclonal antibody against BIGH3 (ab89062, dilution 1: 500, Abcam, MA, USA), and the secondary antibody used was HRP-conjugated goat anti-mouse IgG (SA00001-1, dilution 1: 2000, Proteintech Group, IL, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC (USA). HUVECs were cultured in M199 medium (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), 30 μg/ml endothelial cell growth supplement (ECGS; BD Biosciences, San Jose, CA), 50 U/ml heparin, 25 mM HEPES, 100 U/ml penicillin-streptomycin (Gibco BRL, Grand Island, NY), and 100 U/ml antibiotics-antimycotics (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every three to four days.

2.3. Cell proliferation assay

HUVECs were cultured in M199 supplemented with 30 ng/ml VEGF (R&D systems, Minneapolis, MN). HUVECs were seeded at 2×10^4 cells per well in 96-well plates and allowed to adhere overnight before experiments were conducted. HUVECs were