• Basic Research •

Inhibitory effect of tenomodulin versus ranibizumab on *in vitro* angiogenesis

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Abstract

• AIM: To evaluate anti-angiogenic effect of tenomodulin (TNMD) and ranibizumab on cell proliferation and capillary-like morphogenesis of vascular endothelial cells under the stimulation of vascular endothelial growth factor (VEGF) *in vitro.*

• METHODS: The effects of TNMD and ranibizumab on VEGF-induced proliferation of human umbilical vein endothelial cells (HUVECs) were evaluated by MTT assay, and the effects of TNMD and ranibizumab on capillary-like structures formed by HUVECs under the stimulation of VEGF were examined in culture. Capillary-like morphogenesis of HUVECs was quantitatively evaluated, and total lengths of tube-like structures per field were measured in a masked way.

• RESULTS: HUVECs with both ranibizumab and TNMD protein showed MTT reduction in VEGF-stimulated cell proliferation as expected, while MTT absorbance in the HUVECs with TNMD was significantly declined than that with ranibizumab (*P*<0.01). The capillary-like structures formed by HUVECs were markedly impaired by the presence of both TNMD and ranibizumab in the culture medium. The total length of the capillary-like structures per field was significantly shorter in the medium with TNMD than that of ranibizumab (*P*<0.01). The inhibitory effect of TNMD on tube formation *in vitro* angiogenesis was significantly stronger than that of ranibizumab.

• CONCLUSION: TNMD may have stronger inhibitory effect than ranibizumab on *in vitro* angiogenesis.

• KEYWORDS: tenomodulin; ranibizumab; inhibitory effect; proliferation; angiogenesis

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INTRODUCTION

Neovascular eye diseases such as diabetic retinopathy, central retinal vein occlusion, and wet age-related macular degeneration (AMD) are characteristic of ocular neovascularization, the pathological vascular proliferation that impairs eyesight[1-2]. Neovascular age-related macular degeneration (NVAMD) is a primary cause of blindness in elderly populations among those diseases[3]. The disease is characterized by the abnormal growth of arteries and veins (neovascularisation) in the macula, the leakage of these blood vessels leads to swelling and damage to the macula, resulting in a fibrous scar that cause uncorrectable vision loss[4]. Therapies against NVAMD target new blood vessels. Ranibizumab is one of the most frequently used anti-vascular endothelial growth factor (VEGF) agents injected intravitreally to treat NVAMD[3]. Ranibizumab (also referred to as lucentis) is a humanized recombinant monoclonal antibody fragment (Fab), targeting the inhibition of human VEGF-A. It is combined with the VEGF-A subtype (*i.e.* VEGF110, VEGF121 and VEGF165) with a high affinity, which inhibits the binding of VEGF-A to its receptor VEGFR-1 and VEGFR-2. VEGFA binding to its receptor, leading to the formation of vascular endothelial cell proliferation and angiogenesis, and increased vascular leakage, all of which are thought to be associated with NVAMD progress[5]. Lucentis was shown to be effective in AMD-associated choroidal neovascularization (CNV) compared with photodynamic therapy or no treatment[6-7]. But for the duration and efficiency of treatment, repeated injections intravitreally are inevitable which may result in further safety risks and increased costs of patients. Due to the lack of a long-term convincing body of evidence regarding safety, the systemic safety of intravitreal lucentis repeatedly is still need to be assessed[4]. Previous studies have reported the adverse events including hypertension, stroke and myocardial infarction *etc*[4]. Thus, doctors have been searching for more effective anti angiogenic drugs to prevent intraocular neovascular disorders.

Tenomodulin (TNMD) is a new member of the tumor necrosis factor family[8], which has been identified as a transmembrane

angiogenesis inhibitor[9]. Few studies have confirmed TNMD as an angiogenesis inhibitor, which inhibits vascular endothelial cell proliferation and tube morphology *in vitro*, and suppresses tumorigenesis *in vivo*[10-13]. In our earlier article, we explored the role of TNMD in retinal neovascularization *in vivo*, and concluded that TNMD inhibits pathologic vascular proliferation in the mouse model of oxygen-induced retinopathy[14].

In this study, we would like to recommend TNMD, a more potent anti-VEGF agent by analyzing the inhibitory effect of TNMD versus ranibizumab *in vitro* angiogenesis.

MATERIALS AND METHODS

Materials TNMD (1 mg/mL) was purchased from Abcam (LA, USA). Kept at -20℃, in sterile PH 7.4, 0.01 mol/L phosphate buffered saline (PBS) once reconstituted. TNMD protein is stable at 2℃-4℃ for at least six weeks. The antibody has a strong hydrophobic, high concentrations lead to precipitation, and freeze-thaw cycles can be repeated 2-3 times. Ranibizumab (lucentis injections, 10 mg/mL) was obtained from Novartis, China, stored at 2℃ -8℃ and cannot be frozen.

Methods

Cell culture Human umbilical vein endothelial cells (HUVECs, KG110, KeyGen BioTECH, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) including 10% fetal bovine serum (FBS) (Gibco, USA) in 5% CO_2 at 37℃, media were changed in each 2 to 3d. Cells were used for the experiments between passages 3 and 6, within these passages, HUVECs kept their endothelial characteristics, such as the cobblestone-like morphogenesis.

Human umbilical vein endothelial cell proliferation assay Cellular proliferation was determined using MTT assay, which was described previously[10]. Briefly, HUVECs at passages 3-6 were harvested with trypsin (KeyGen BioTECH, China) and suspended in DMEM at a density of 50 000 cells/mL. The cells were seeded into 96-well (Corning, USA) microplates (100 μL per well) and grown for 24h. The cells were then starved in FBS free culture medium for 6h and stimulated with VEGF (Sino Biological Inc., China) or VEGF with lucentis (0.25, 0.5, 1, 2 μg/mL) or VEGF with TNMD (0.25, 0.5, 1, 2 μg/mL) respectively at the indicated concentrations for 12h. After the stimulation, 10 μL of MTT at 5 mg/mL (Amresco, USA) was put into each well, and the cells were then incubated for another 4h. After 150 μL of dimethyl sulfoxide (DMSO) (Applichem, Germany) was added and mixed thoroughly for 10min, optical density was then measured by a microplate reader (Themo Multiscan MK3, USA) at 490 nm.

Matrigel tube formation assay The 24-well tissue culture plates (Corning, USA) were coated with Matrigel Matrix (400 μL per well, BD, USA) and incubated at 37℃ for 30min. HUVECs starved in 1% FBS containing culture medium for 4h were harvested with trypsin and were seeded at a density of 60 000 cells per well on polymerized Matrigel in the existence of VEGF (100 ng/mL) or VEGF with lucentis and VEGF with TNMD at the indicated concentrations. Cells were also seeded in 10% FBS containing culture medium as a positive control. The plate was incubated at 37℃ for 6h and then photographed (Olympus IX81, Japan). To quantitatively assess the capillary-like morphogenesis of HUVECs, total lengths of capillary-like structures per field were measured in a masked way, using image processing and analysis software (Image J software, National Institutes of Health, USA, NIH Image J Version 1.61, acquired from the public domain http://rsb.info.nih.gov/nih-image *via* the National Institute of Health, Bethesda, MD, USA). Each experiment was performed at least 3 times.

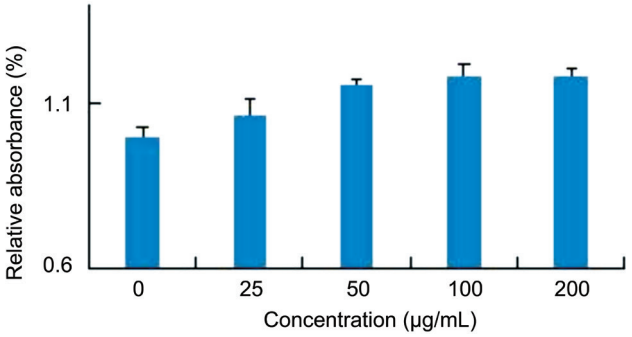

Figure 1 VEGF screened for optimum concentration of cell proliferation.

Statistical Analysis Each experiment was done at least thrice, and the data were statistically analyzed by using SPSS 13.0, one-way ANOVA, followed by Scheffe's multicomparison test. *P* value of <0.05 was considered statistically significant.

RESULTS

Vascular Endothelial Growth Factor Screened for Optimum Concentration of Cell Proliferation HUVECs were seeded into 96-well microplates (100 μL per well) and grown for 24h. The cells were then starved in FBS free culture medium (0.5% FBS) for 6h and stimulated with VEGF of different concentration (25, 50, 100, 200 ng/mL) for another 24h. VEGF-induced endothelial proliferation was evaluated by measurement of MTT assay. HUVECs were significantly stimulated by VEGF at the indicated concentration, up to 100 ng/mL (P<0.01) (Figure 1).

Comparing Suppressive Effect of Tenomodolin/Lucentis on Vascular Endothelial Growth Factor-induced Endothelial Proliferation The suppressive effect of TNMD and lucentis on VEGF-induced endothelial proliferation was assessed by measurement of MTT assay. HUVECs were significantly stimulated by VEGF at the indicated concentration, up to 100 ng/mL (Figure 1). HUVECs with both TNMD and lucentis protein showed MTT reduction in VEGF-stimulated cell proliferation as expected, in contrast, MTT absorbance in

the HUVECs with TNMD significantly declined than that with lucentis (P<0.01) (Figure 2).

Comparison Inhibitory Effect of Lucentis/Tenimodulin on Vascular Endothelial Growth Factor-mediated Human Umbilical Vein Endothelial Cell Tube Formation *in Vitro* Angiogenesis To compare the suppressive effects of lucentis and TNMD *in vitro* angiogenesis, capillary like morphogenesis of HUVECs was evaluated by culturing in various conditioned media. HUVECs were flated on the matrix in the existence of 100 ng/mL VEGF. The capillary-like structures formed by HUVECs were markedly impaired by the existence of both TNMD and lucentis in the culture medium. The total length of the capillary-like structures in each field was significantly shorter in the medium with TNMD than that of lucentis (Figure 3). The inhibitory effect of TNMD on tube formation *in vitro* angiogenesis was significantly stronger than that of lucentis (P<0.01).

DISCUSSION

Neovascular eye disease is a main cause of severe vision loss at present worldwide. The treatment of intraocular neovascular disease is being innovated by intravitreal therapies targeting VEGF[15]. Intravitreal injection anti-VEGF agents, aim to prevent the growth of abnormal blood vessels in the eye to stop vision loss and, in some cases, improve vision. Although ranibizumab as an anti-VEGF agent is one of the most frequently used anti-VEGF drugs injected intravitreally to treat wet AMD[3], and has been proved to be effective with respect to preserving or improving visual acuity, the major eye adverse events detected in clinical tests such as a low frequency of ocular inflammation, a slightly elevated risk of monocular hemorrhage, stroke[15] and so on keep exist. High cost is also a problem need to be concerned in developing countries.

Recently, many research labs have been trying to better understand the molecular mechanisms of the occurrence of neovascularization and possibilities for recovery from retinopathy or maculopathy. Currently, a lot of new protein class molecules with important regulatory functions have been discovered and identified[16].

TNMD, a more potent anti-VEGF agent, primarily expressed in dense hypovascular connective tissues such as tendon, ligament, and sclera, vitreous body of eye[17-19]. Three-fold higher *TNMD* gene expression levels have been observed in adipocytes and adipose tissue as compared to other human tissues[20]. Jelinsky *et al*[21] reported that the TNMD expression was four times higher in tendons than in the adipose tissue, moderate TNMD expression is demonstrated in cartilages and bones. TNMD has various biological functions. Tolppanen *et al*[22] summarized that TNMD could have genetic associations with the central obesity, inflammations, serum level of system immune mediators, AMD, Alzheimer disease, type 2 diabetes,

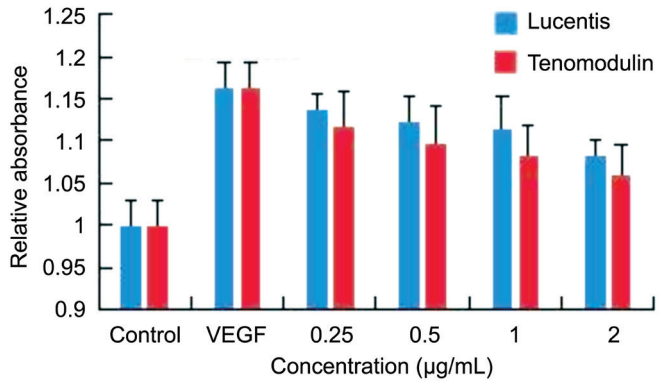

Figure 2 Inhibitory effect of TNMD and lucentis on VEGF-induced endothelial proliferation The suppressive effect of TNMD and lucentis on VEGF-induced endothelial proliferation was evaluated by measurement of MTT assay.

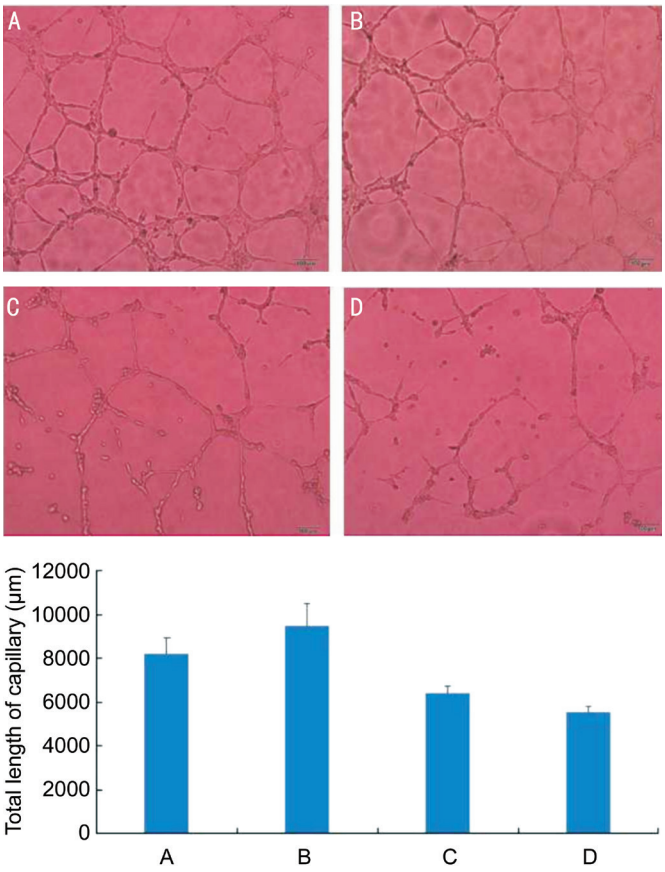

Figure 3 Suppressive effect of TNMD/lucentis on VEGF-induced endothelial proliferation and on *in vitro* angiogenesis (×100) The tube morphogenesis of HUVECs in different conditioned culture medium. A: 1% FBS containing medium as positive control; B: Medium including 1% FBS and 100 ng/mL VEGF; C: Medium including 1% FBS, 100 ng/mL VEGF and lucentis 1 μg/mL; D: Medium including 1% FBS, 100 ng/mL VEGF and TNMD 10 μg/mL. Bar chart: quantitative assessment of the length of the capillary structures by image computer analysis. Data are means±SD of three independent experiments. The total length of HUVECs incubated in the medium containing TNMD had significant differences with that of in the other three different conditioned culture medium (P<0.01).

glucose and lipid metabolism. TNMD is one of the most downregulated genes in patients with metabolic syndrome symptoms, impaired fasting glycaemia and weight reduction intervention[23]. TNMD also plays a crucial role in cardiac valve tissues degeneration by control of the angiogenesis and the matrix metalloproteinase synthesis[24]. It has been reported that TNMD inhibits proliferation and tube morphogenesis of vascular endothelial cells *in vitro* and has a potent anti-tumor effect *in vivo*. Clinical and laboratory studies have also reported strong evidence indicating that tumor angiogenesis is inhibited by administrating anti-angiogenic inhibitory factors. Our earlier study has reported that it is effective in preventing ischemic-induced retinopathy and pathologic angiogenesis[14] when TNMD be injected in the vitreous body of C57BL/6 mice with an oxygen-induced retinopathy.

In this study, we analyzed the inhibitory effect of ranibizumab versus TNMD *in vitro* angiogenesis by comparing the anti-angiogenic effect of TNMD and lucentis protein on cell proliferation and capillary-like morphogenesis of vascular endothelial cells under the stimulation of VEGF *in vitro*. HUVECs with both lucentis and TNMD protein showed MTT reduction in VEGF-stimulated cell proliferation as expected, in contrast, MTT absorbance in the HUVECs with TNMD significantly declined than that with lucentis. The capillary-like structures formed by HUVECs were markedly impaired with the culture medium containing both TNMD and lucentis. The total length of the capillary-like structures in each field was significantly shorter in the medium with TNMD than that of lucentis (Figure 2). The inhibitory effect of TNMD on tube formation *in vitro* angiogenesis was significantly stronger than that of lucentis.

In conclusion, these results indicate that TNMD may have stronger inhibitory effect than ranibizumab on *in vitro* angiogenesis. This is an interesting finding and also a relatively shallow study that further research and confirmation on TNMD such as toxicity, safety check, duration of action, *etc*. is necessary. The observations may provide us with a more effective and better role in the treatment of pathologic neovascular conditions in the near future.

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