·Review·

Recent advances on the modified endostatin and ocular neovascularization

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Abstract

• Endostatin (ES), the C-terminal fragment of collagen XVIII, is a potent angiogenesis inhibitor. At present, there are a large number of research papers on ES. It has already been on clinical stage Ⅱ and been widely used in inhibition of neovascularization (NV). However, how to improve the bioactivity of ES is still a matter of ongoing discussion. The objective of this review is to elucidate the relationship between the modified ES and ocular neovascualrization, and to discuss the superiority based on the structure modification. The structure can be changed either by covalent modification or by genetic mutation. It is proposed that the secondary structral ES enhance the anti-angiogenic activity. Studies on modified ES also shed light on our understanding of the molecular action mechanisms of ES. Modified ES may be exploited as a new angiogenesis inhibitor for therapeutic applications, in substitution of the native ES.

• KEYWORDS: modified endostatin; ocular neovascular-ization; antiangiogenesis activity

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INTRODUCTION

Most of diseases that cause catastrophic loss of vision can be blamed to ocular neovascularization (NV), such as corneal NV, diabetic retinopathy (DR) and age-related macular degeneration (AMD). The exact pathogenicity of ocular NV is not yet well understood, and there is no satisfactory therapy for ocular NV. Vascular endothelial cells migrate and proliferate to form new blood vessels. Endostatin (ES) has been characterized and was identified by its ability to inhibit endothelial cell proliferation, migration and cord formation and to suppress angiogenesis [1]. It is believed to be promising in the treatment of ocular NV in the near future. We reviewed recent progress in studies on the mechanisms and therapeutic potential of modified ES in ocular NV.

STRUCTURE OF ES

ES was first identified in the conditioned medium of hemangioendothelioma cells by O'Reilly *et al* ES is derived from the non-triplehelical C-terminal NC1 domains of collagens XVIII, which is released proteolytically in trimeric form and further converted to monomeric ESs of about 20kDa. The fragment has been characterized with antiangiogenic properties [2]. X-ray diffraction demonstrated ES possesses a compact globular folding and a core structure related to the carbohydrate recognition domain of C-type lectins [3]. Analogous to many other angiogenesis inhibitors, ES has a strong affinity for heparin. Two heparin-binding domains have been identified in ES involving two clusters of arginine residues [4], and a zinc binding site is located in the N-terminal part of the molecule[5]. The possibility that its anti-angiogenic effect might be related to displacement of angiogenic factors from the surface of endothelial cells through binding of heparan sulfate has prompted several investigations of its interaction with heparin and heparan sulfate (HS)[6]. The role of zinc in the biological activity of ES remains controversial. Zinc-binding has been reported to be essential for the anti-angiogenic activity of ES [7]. Later studies have failed to confirm the relationship between zinc-binding and inhibition of endothelial cell migration or angiogenesis[8].

MECHANISMS OF ES

Receptor Pathway Evidence suggests that ES binds to cell surface receptors, such as vascular endothelial growth factor (VEGF), integrins, HS, nucleolin receptor. Kim *et al*[9] demonstrated that ES binds directly to VEGF receptors but

not to VEGF, and that binding of ES to VEGF receptor blocks VEGF-induced tyrosyl phosphorylation of VEGF receptors (KDR/flk-1), MAP kinases, and FAK in human umbilical vein endothelial cells. Rehn *et al* [10] demonstrated that soluble ES binds to integrin α5 and αv to inhibit human vascular endothelial cell migration. Javaherian *et al* [11] demonstrated that oligomeric ES binds to HS on the cell surface to regulate migration and morphogenesis of vascular endothelial cells. Shi *et al* [12] found that ES is internalized and transported into cell nuclei of endothelial cell via nucleolin. The phosphorylation of nucleolin, which is critical for cell proliferation, can be inhibited by ES in the nucleus.

Multiple Mechanisms The rest of multiple mechanisms for ES functioning were characterized as follows: ① ES inhibits vascular endothelial tube formation by inhibiting nitric oxide synthase; ② ES induces endothelial cell apoptosis by activating caspase-3 enzymatic activity, reducing antiapoptotic protein BcL-2, and reducing MAP kinases activities; ③ ES regulates the Wnt signaling pathway by promoting β catenin degradation; ④ ES causes G1 arrest of endothelial cells and down-regulating c-myc mRNA expression and decreasing the mRNA and protein of cyclin D1[13].

ES AND OCULAR NV

ES was found universal expression in ocular structure, namely the basement membranes (BMs) of the corneal and conjunctival epithelia, the BMs of the pigment epithelium of the retina, and the internal limiting membrane and so on. The ubiquitous distribution of ES in human ocular tissues may be related to the avascularity of the eye[14].

ES exerts powerful anti-angiogenic effect and without the development of resistance and toxicity, therefore it is drawing more and more ophthalmologist's attention. ES can be administrated by topical instillation, subconjunctival injection, intra-vitreous injection and gene therapy. Gene transfer provides a strategy to achieve sustained release of ES and can circumvent difficulties arising from handling the protein. The effect of intraocular delivery of recombinant viruses carrying genes encoding angiostatic proteins has been demonstrated in experimental models of ocular NV[15]. Lai *et al* [16] used a recombinant adeno-associated viral (rAAV) vector carrying ES gene to examine the inhibition of corneal NV induced by silver nitrate cauterization in mice. They concluded the rAAV was capable of directly delivering genes to the ocular surface epithelium by way of subconjunctival injection and was able to deliver sustained high levels of gene expression *in vivo* to inhibit angiogenesis. Zhang *et al* [17] subconjunctively injected pBlast-hES to investigate gene therapy of rat corneal NV induced by acid cauterization.

MODIFICATION OF ES

There are lots of obstacles on ES's clinical application, such as need of high dose to maintain its efficacy, poor stability, etc. In order to overcome these shortcomings, structural modification has received particular attention. Many scholars reformed ES to enhance the stability and improve targeted therapy of ES, these measures indeed improve the antiangiogenic ability of ES.

Chemical Modification Polyethylene glycol (PEG) is a highly investigated polymer for the covalent modification of peptides and proteins. PEG possesses superiorities in shielding antigenic and immunogenic epitopes in compared with other modifiers [18]. PEG shows better amphipathic properties and biocompatibility. Li *et al* [19] first applied polyethylene glycol ES (PEG-ES) to inhibit corneal NV induced by alkali burn in experimental model, the result demonstrated that PEG-ES possesses more anti-angiogenic activities than ES, and there were no toxicity and adverse reactions when local administrated.

The chemical modification of ES with low molecular weight heparin (LMWH) is mainly based on the sequence of LMWH sugar molecules exist two-o-hydroxy structure, after the periodic acid oxidation a highly active aldehyde can be formed, which combined with the free amino of ES protein to form covalent modification of ES. Tan *et al* [20] modified ES by LMWH (LMWH-ES), the changes of the secondary structure of the modified products were studied by Fourier transform infrared spectroscopy and Circular dichroism spectra. Their study demonstrated that the modified products have a better heat tolerance and higher activity than ES towards. Zhu *et al* [21] first administrated LMWH-ES by subconjuctival injection in rabbit corneal NV model, the result showed that LMWH-ES was superior to ES in the inhibition of NV.

Genetic Modification

P125A Recent studies have shown that a point mutation in human ES at position 125 can obtain a mutant ES, called P125A-ES (P125A-ES)[22]. This genetically engineered ES

showed improved endothelial cell binding and antiangiogenic biological activity when compared to the native protein. P125A-ES can be acquired through genetically replaced the amino acid proline at position 125 with alanine.

RGD sequence Neovascular tissue express high levels of αvβ3/avβ5 and a5β1 integrins. Consequently, peptides containing the RGD (Arg-Gly-Asp) sequence, which is present in ligands of integrins, is effective in targeting therapeutic reagents to neovascular endothelium [23]. Yokoyama *et al* [24] added RGD sequence to either the amino or carboxyl terminus of P125A-ES to get further modification of P125A-ES with the RGD motif. RGD-modified P125A-ES showed increased binding to endothelial cells and improved antiangiogenic properties. Ren *et al* [25] changed GRIRGAD sequence of ES into RGDRGD by the method of site-directed mutagenesis to raise its anti-angiogenic activity.

NGR motif Human ES has an internal asparagine-glycine-arginine (NGR) motif at position 126-128. Peptides that contain NGR sequence have been shown to target tumor vasculature and inhibit aminopeptidase N activity [26]. Yokoyama *et al*added NGR sequence to the amino terminus of the human ES through genetical modification, NGR-ES showed improved inhibition of tumor growth, endothelial cell homing and biologic activity.

Endostar Endostar, a novel recombinant human ES, was purified in Escherichia coli with an additional nine-amino acid sequence (MGGSHHHHH)[27]. The protein can be folded into a soluble one, the antiangiogenic effects of endostar were correlated with the VEGF-triggered signaling [28]. Endostar suppressed the VEGF-stimulated proliferation, migration, and tube formation.

CONCLUSION

Recent studies demonstrated that modification of a vascular targeting sequence to enhance the biology characteristics and therapeutic value of human ES is encouraging. However, its action mechanisms have not been fully elucidated. The relationship between the structure and function of ES warrant further investigation, so as to explore new substitution with native ES. Most of the modified ES have not been used in ocular diseases yet, we are longing for more and more administration in ophthalmology fields.

Gene transfer provides a means to treat ocular NV without the development of toxicity and tolerance. Modified ES in combined with gene therapy may be harnessed to provide us broader prospects in the treatment of ocular NV.

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