Biomaterials 112 (2017) 218–233

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Anti-angiogenesis through noninvasive to minimally invasive intraocular delivery of the peptide CC12 identified by in vivo-directed evolution

Chong Chen [a], Kun Liu [a], Yupeng Xu [a], Pengwei Zhang [b], Yan Suo [a], Yi Lu [a], Wenyuan Zhang [c], Li Su [a], Qing Gu [a], Huamao Wang [b], Jianren Gu [b], Zonghai Li [b, **], Xun Xu [a, *]

[a] Department of Ophthalmology, Shanghai Key Laboratory of Fundus Disease, Shanghai General Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200080, PR China
[b] State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital Affiliated to Medical School of Shanghai Jiao Tong University, Shanghai 200032, PR China
[c] Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China

ARTICLE INFO

Article history:
Received 11 June 2016
Received in revised form
20 September 2016
Accepted 29 September 2016
Available online 30 September 2016

Keywords:
Angiogenesis inhibitor
Cell-penetrating peptides
Intraocular drug delivery
In vivo-direction evolution
Neovascularization
Phage display

ABSTRACT

Anti-vascular endothelial growth factor (VEGF) therapies are widely used for the treatment of neovascular fundus diseases such as diabetic retinopathy. However, these agents need to be injected intravitreally, because their strong hydrophilicity and high molecular weight prevent them from penetrating cell membranes and complex tissue barriers. Moreover, the repeated injections that are required can cause infection and tissue injury. In this study, we used *in vivo*-directed evolution phage display technology to identify a novel dodecapeptide, named CC12, with the ability to penetrate the ocular barrier in a noninvasive (via conjunctival sac instillation) or minimally invasive (via retrobulbar injection) manner. KV11, an antiangiogenesis peptide previously demonstrated to inhibit pathological neovascularization in the retina, was then used as a model antiangiogenesis cargo for CC12. We found that conjugation of KV11 peptide with CC12 peptide facilitated the delivery of KV11 to the retina, resulting in significant inhibition of retinal neovascularization development via topical application without tissue toxicity. Collectively, our data of multilevel evaluations demonstrate that CC12 may enable the noninvasive to minimally invasive intraocular delivery of antiangiogenic therapeutics.

1. Introduction

Neovascular eye diseases such as age-related macular degeneration (AMD) and diabetic retinopathy (DR) have become the leading cause of blindness in developed countries, as well as in China [1,2]. Intravitreal injection of biological drugs, such as the widely used anti-vascular endothelial growth factor (VEGF) recombinant monoclonal antibodies (e.g., ranibizumab, 48 kDa) or VEGF decoy receptor fusion proteins (e.g., Conbercept, 143 kDa), provides a potent prevention and treatment modality for wet-AMD and DR [3,4]. However, this is an invasive strategy which can be exacerbated with repeated injections during the long-term therapy of neovascular fundus diseases [5,6], may associate with patient noncompliance and inevitably cause complications such as cataract formation, increased intraocular pressure, retinal detachment, vitreous hemorrhage, endophthalmitis and tissue injury [7].

Additionally, in physiological conditions, large molecules (molecular weight higher than 40 kDa for linear molecules or 70 kDa for spheroid molecules) are difficult to diffuse the internal limiting membrane to reach the outer retina and choroid by intravitreal administration [8]. And the weights and strong hydrophilicity of large biological molecules make it difficult to penetrate the ocular obstacles through conjunctival sac instillation. Besides, the sclera is permeable to molecules as large as 70 kDa via porous diffusion [8], and the retina is permeable to molecules as large as 76.5 kDa [9]. The biological barriers such as blood-retinal barrier together with

* Corresponding author. No.100, HaiNing Road, Shanghai 200080, PR China. Tel.: +86 21 63240090; fax: +86 21 63067385.
** Corresponding author. No.25/Ln2200, XieTu Road, Shanghai 200032, PR China. Tel.: +86 21 64436601; fax: +86 21 64432027.
E-mail addresses: zonghaili@163.com (Z. Li), drxuxun@sjtu.edu.cn (X. Xu).

http://dx.doi.org/10.1016/j.biomaterials.2016.09.022
0142-9612/

the functional presence of blood-aqueous barrier make drug delivery to the posterior segment a very challenging issue [10].

It's reported that the KV11 peptide (~2 kDa) [11] could reduce the pathologic neovascularization in a mouse model via intravitreal injection, without affecting normal retinal vasculature or its function. If KV11 peptide could penetrate from outside of the eyeball to reach the inner retina with the aid of a carrier molecule or noninvasive intraocular delivery system, it may provide a promising alternative or a beneficial supplement for antiangiogenic therapy in a lot of fundus diseases.

Regarding drug delivery system, researchers are increasingly investigating whether small-molecule cell-penetrating peptides (CPPs) [12,13], adeno-associated viral vectors [14], mucoadhesive delivery systems [15] and nanoparticle drug carriers [16] could be used to enhance the intracellular or *in vivo* drug delivery [17]. Viral- or non-viral vectors mediated gene transfer has been widely concerned in ocular administration and provide promising results, however, some studies have pointed out that over-expression of therapeutic genetic products into the eye may be deleterious [13]. What is more, the limitations of viral vector also include low transfected efficiency together with cellular toxicity.

Whereas, in terms of CPPs, they have the following outstanding advantages as candidates for the treatment of neovascular eye diseases: low molecular weight and relatively simple structure, which means they can be formed by relatively inexpensive *in vitro* solid-phase chemical synthesis, and chemical modification is thus convenient; highly stable, which means they require less temperature regulation to maintain a certain biological activity, so it would be convenient for storage and would be portable if made into eye drops; high ocular tissue permeability; low toxicity and immunogenicity [18]. Besides, CPPs have demonstrated an effective ability to overcome various biological barriers at the tissue level, including the blood-brain barrier [19], the skin [20], the nasal mucosa [21], the intestinal walls [22], and even the intratumoral barrier [16]. Thus, the research focus of drug delivery has shifted from intracellular delivery to *in vivo* delivery [17]. Therefore, small-molecule peptide inhibitors of neovascularization conjugated with CPPs would be highly effective drugs in the clinical treatment of neovascular eye diseases [23].

Although the well-known CPPs such as HIV trans-activating factor (TAT) and HSV VP22 act efficiently *in vitro*, their performance in penetrating the retina *in vivo* has proven to be limited [13,24,25]. Furthermore, TAT-based modification can lead to endocytosis and a lack of cell specificity, which in turn accelerates elimination through the mononuclear phagocyte system [26–28]. Recently, researchers investigated the potential use of penetratin and other CPPs both *in vitro* and *in vivo* and found that penetratin may be a potentially powerful ocular absorption enhancer [29]. However, that study did not establish whether penetratin could mediate the uptake of other therapeutic molecules or drugs across the ocular barrier to elicit a therapeutic effect. Moreover, the nonselective penetration of CPPs often causes unwanted wide drug distribution [30]; therefore, a screening method that is based on retinal affinity would be a potential solution for achieving targeted delivery.

In this study, we used *in vivo*-directed evolution phage display technology to identify a novel peptide that could enable a needle-free, long-lasting and cost-effective ocular delivery platform. The high-throughput biopanning nature of the process provides a rapid way to screen recombinant phages that might possess high penetration capability through the ocular impediments. *In vivo*-directed evolution involved the topical conjunctival sac instillation or retrobulbar injection of a peptide library into rats. Although retrobulbar injection still involves a minimal invasive operation, it is more widely accepted by patients in clinical practice over intravitreal injection. And retrobulbar/peribulbar administration is deemed to provide prolonged pharmacologic impacts with lower toxicity [6].

Using this approach, we identified a new peptide for ocular delivery (POD) named CC12, which was selected for further evaluation. And we found that the CC12-mediated therapeutic peptide KV11 penetrated the retinal layers via topical application; what's more, the conjugated peptide CC12-KV11 maintained the anti-angiogenesis effect without apparent toxicity, indicating that CC12 could enable the noninvasive to minimally invasive, long-term treatment of neovascular disease across the entire retina.

2. Materials and methods

2.1. Materials and reagents

The dodecapeptide Phage Display Peptide (Ph.D.-12™) library (complexity of the library on the order of 10^9 independent clones), *Escherichia coli* ER2738 and phage culture reagents were obtained from Raygene Biotechnology (Shanghai, China) as a gift.

All the candidate peptides and control peptides were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China).

Human Retinal Pigment Epithelium Cell Line (ARPE-19) and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Maryland, USA) and cryopreserved in the tissue culture laboratory at the Institute of Ophthalmology, Shanghai General Hospital (Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Rockville, MD, USA). Colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA).

Propidium iodide (PI) and Alexa Fluor 568-conjugated isolectin B_4 were purchased from Molecular Probes (Eugene, OR, USA).

^{125}I was purchased from and the isotope tracer experiment was carried out in the Department of Radioactive Pharmaceutical Sciences at Fudan University (Shanghai, China), which has an isotope state-issued operating license and import isotopes license.

Fluorescein isothiocyanate (FITC) recombinant human $VEGF_{165}$ and heparan sulfate were obtained from Sigma-Aldrich (St Louis, MO, USA). Growth Factor Reduced Matrigel was purchased from BD (Franklin Lakes, NJ, USA). Ranibizumab (Lucentis®) was purchased from Novartis (Basel, Switzerland). Transwell chambers were purchased from Corning (Corning, NY, USA).

All chemicals used were of analytical grade.

2.2. Animals

All animal experiments were approved by the Medical Ethics Committee of the Shanghai General Hospital Affiliated to Shanghai Jiao Tong University and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (0.15 kg), neonatal C57BL/6J mice with mothers, and adult albino rabbits (2.5 kg) were provided by the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences. The animals were housed in an air-conditioned room on a 12 h light–dark cycle with access to food and water *ad libitum*. The animals were acclimatized to laboratory conditions for 1 week prior to the experiments. Fertilized chicken embryos (postnatal 1–2 days) were purchased from the Academy of Agricultural Sciences, Shanghai, China.

2.3. Phage library selection and evolution

Phage display (Ph.D.™) is a technology that combines gene