Acta Biomaterialia xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Short peptide analogs as alternatives to collagen in pro-regenerative corneal implants

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ARTICLE INFO

Article history:
Received 19 June 2017
Received in revised form 8 January 2018
Accepted 10 January 2018
Available online xxxx

Keywords:
Collagen-like peptide
Recombinant human collagen
Cornea
Regeneration
Exosomes

ABSTRACT

Short collagen-like peptides (CLPs) are being proposed as alternatives to full-length collagen for use in tissue engineering, on their own as soft hydrogels, or conjugated to synthetic polymer for mechanical strength. However, despite intended clinical use, little is known about their safety and efficacy, mechanism of action or degree of similarity to the full-length counterparts they mimic. Here, we show the functional equivalence of a CLP conjugated to polyethylene glycol (CLP-PEG) to full-length recombinant human collagen *in vitro* and in promoting stable regeneration of corneal tissue and nerves in a pre-clinical mini-pig model. We also show that these peptide analogs exerted their pro-regeneration effects through stimulating extracellular vesicle production by host cells. Our results support future use of CLP-PEG implants for corneal regeneration, suggesting the feasibility of these or similar peptide analogs in clinical application in the eye and other tissues.

Statement of significance

Although biomaterials comprising full-length recombinant human collagen and extracted animal collagen have been evaluated and used clinically, these macromolecules provide only a limited number of functional groups amenable to chemical modification or crosslinking and are demanding to process. Synthetic, customizable analogs that are functionally equivalent, and can be readily scaled-up are therefore very desirable for pre-clinical to clinical translation. Here, we demonstrate, using cornea regeneration as our test bed, that collagen-like-peptides conjugated to multifunctional polyethylene glycol (CLP-PEG) when grafted into mini-pigs as corneal implants were functionally equivalent to recombinant human collagen-based implants that were successfully tested in patients. We also show for the first time that these materials affected regeneration through stimulation of extracellular vesicle production by endogenous host cells that have migrated into the CLP-PEG scaffolds.

1. Introduction

The current global need for replacement organs and tissues requires numbers that far exceed the donor supply. Artificial organs as alternatives can potentially save and improve the quality of lives of patients. The macromolecules of the extracellular matrix (ECM), specifically collagen, have been extensively tested for use as organ substitutes [1]. The premise is that the ECM macromolecules

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https://doi.org/10.1016/j.actbio.2018.01.011
1742-7061/

will stimulate regeneration, recapitulating their role in organogenesis during embryogenesis. ECM-derived or inspired biopolymers ranging from decellularized tissues/organs to fabricated constructs are being evaluated or used clinically for promoting regeneration. However, natural ECM macromolecules like collagen are large and difficult to extract and process or modify, and are heterogeneous and source-dependent. While the use of recombinantly produced human collagen eliminates heterogeneity, these are still large macromolecules that require significant processing. This makes short peptide analogs that are readily prepared, easily customized and scaled-up, very attractive ECM alternatives for regenerative medicine. Several *in vitro* and *in vivo* studies have reported that such short ECM-mimicking peptides can stimulate regeneration in a range of organ systems including bone and spinal cord [2,3]. However, despite their great potential, little is known about their safety and efficacy, mechanism of action, or functional equivalence to their clinically evaluated full-length counterparts.

Here, we tested under pre-clinical conditions in mini-pigs, the safety and efficacy of a collagen-like peptide (CLP) conjugated to an inert but mechanically tougher multifunctional polyethylene glycol (PEG) to emulate the function of a collagen-based implant [4] for promoting regeneration, using the cornea as a model system. The cornea is the transparent front of the eye and major refractive surface for focusing light to the retina for vision. Its superficial location and transparency allow easy access and real-time visualization of cell-biomaterial interactions. A diseased or damaged cornea with permanent transparency loss results in blindness. The conventional treatment is transplantation with donated human corneas. However, like other organs, cornea transplantation suffers from a severe global shortfall, with an estimated 12.7 million patients worldwide awaiting transplantation, or only one out of every 70 patients being transplanted [5]. The transparency, accessibility plus the pressing medical need make the cornea an attractive model tissue.

We previously used full-length recombinant human collagen type III (RHCIII) to fabricate implants that successfully stimulated stable regeneration of the human cornea, an organ that does not normally regenerate on its own. Corneal implants comprising RHCIII only and RHCIII incorporating 2-methacryloyloxyethyl phosphorylcholine (MPC), a synthetic lipid-polymer that can suppress inflammation, [6] were evaluated clinically [7–9]. They respectively promoted stable corneal regeneration without continual immunosuppression in 10 conventional transplantation patients [7,8] and three patients diagnosed as being at high-risk for rejection of donor corneas [9]. Although the use of RHCIII instead of extracted animal collagen mitigates potential source heterogeneity, xenogeneic reaction, or risk of pathogen transmission [10,11], RHCIII replicates full-length collagen, which, is large and relatively difficult to chemically tailor or process, unlike short peptides. In this study, we compared in detail the safety, efficacy, and mechanism of promoting regeneration of corneal implants made from CLP-PEG to control implants made from clinically tested RHCIII-MPC *in vitro*, and in rabbit and mini-pig animal models. Successful testing in a simple organ system like the cornea will allow for the extension to more complex applications such as skin and heart, as we have shown with collagen after minimal modification [12–14].

2. Materials and methods

2.1. Hydrogel implants

CLP-PEG and RHCIII-MPC implants were prepared as previously described [4,15]. Briefly, CLP comprising Cys-Gly-(Pro-Lys-Gly)$_4$(Pro-Hyp-Gly)$_4$(Asp-Hyp-Gly)$_4$, was synthesized (UAB Ferentis, Vilnius, Lithuania) and conjugated to a 40 kDa 8 arm PEG-maleimide (Creative PEG Works, NC, USA) by continually stirring in ddH_2O at pH 4.5 for 2 days at a molar ratio of 32:1 and then dialyzed against distilled water using 12–14 kDa MWCO tubing for 2–3 days before lyophilization. 500 mg of 12% (w/w) CLP-PEG solution prepared was crosslinked with EDC and NHS at molar equivalents of CLP-PEG-NH_2:EDC (1:2) and at equal molar ratio of EDC: NHS. All the reagents were moulded into flat sheets or cornea-shaped implants [4].

RHCIII-MPC implants were fabricated by mixing 18% (w/w) aqueous solution RHCIII (FibroGen Inc., San Francisco, CA) with 2-methacryloyloxyethyl phosphorylcholine (MPC, Paramount Fine Chemicals Co. Ltd., Dalian, China) and poly(ethylene glycol) diacrylate (PEGDA, Mn 575, Sigma-Aldrich) in a morpholinoethane sulfonic acid monohydrate (MES, Sigma-Aldrich, MO) buffer. The ratio of RHCIII:MPC was 2:1 (w/w) and PEGDA: MPC was 1:3 (w/w). Polymerization initiators ammonium persulphate (APS; Sigma-Aldrich) and N,N,N,N-tetramethylethylenediamine (TEMED, Sigma-Aldrich) at ratios of APS:MPC = 0.03:1 (wt/wt), APS:TEMED (wt/wt) = 1:0.77, crosslinker, N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide (EDC; Sigma-Aldrich) and its co-reactant, N-hydroxysuccinimide (NHS; Sigma-Aldrich) was then mixed in. The resulting solution was dispensed into cornea-shaped moulds and cured [15].

After demoulding, all hydrogels were washed thoroughly with phosphate buffered saline (PBS) and placed into vials of aseptic PBS containing 1% chloroform, which were sealed to maintain sterility.

2.2. Physical and mechanical characterization

Flexure tests, physical appearance and light transmittance measurements were carried out following the established quality control (QC) protocols [15,16]. The same tests were used to determine stability of the implant after storage for a minimum of 12 months at 4 °C. Qualitative analysis included Fourier Transform Infrared (FTIR) spectroscopy to evaluate the chemical integrity of the components within the hydrogel implants after 12 months of storage. The most relevant amide IR vibrations for CLP were used for this purpose: Amide A, B, I and II [17]. Samples were dried for 3 days. Measurements were carried out in a Nicolet 6700 FTIR spectrometer equipped with a Smart iTR Attenuated Total Reflectance (ATR) sampling accessory with 4 cm^{-1} resolution; a total of 64 individual spectra were collected for each sample. Representative spectra were collected in a Nicolet iS5 FT-IR spectrometer equipped with an iD7 ATR accessory using 300 individual spectra with 4 cm^{-1} resolution.

Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, MO, USA), at 5 U/ml in 0.1 M Tris-HCl buffer containing 5 mM $CaCl_2$ was used to evaluate the stability of hydrogels as previously described [4]. Briefly, samples were weighed after blotting off surface water at different time points to determine the rate of loss of mass. The percentage of residual weight was calculated using the following equation: Residual mass % = W_t/W_o%, where W_t is the weight of hydrogel at a certain time point and W_o is the initial weight of the hydrogel.

Denaturation temperature of the CLP-PEG hydrogels was determined using a Q2000 differential scanning calorimeter (TA Instruments, New Castle, DE), with a temperature range from 8 to 200 °C at a scan rate of 5 °C min^{-1}. Hydrogels (approximately 3 to –5 mg) were placed in an aluminum pan after removing the surface water. The pan was then hermetically sealed to make an airtight condition. Denaturing temperature was calculated from the curve of heat flow versus temperature increase.

The refractive indices of CLP-PEG hydrogels were measured at 19 °C at a wavelength of 589 nm, using a bench-top Abbe 60 series

Please cite this article in press as: J.R. Jangamreddy et al., Short peptide analogs as alternatives to collagen in pro-regenerative corneal implants, Acta Biomater. (2018), https://doi.org/10.1016/j.actbio.2018.01.011