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The promotion of functional urinary bladder regeneration using anti-inflammatory nanofibers

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ABSTRACT

Current attempts at tissue regeneration utilizing synthetic and decellularized biologic-based materials have typically been met in part by innate immune responses in the form of a robust inflammatory reaction at the site of implantation or grafting. This can ultimately lead to tissue fibrosis with direct negative impact on tissue growth, development, and function. In order to temper the innate inflammatory response, anti-inflammatory signals were incorporated through display on self-assembling peptide nanofibers to promote tissue healing and subsequent graft compliance throughout the regenerative process. Utilizing an established urinary bladder augmentation model, the highly pro-inflammatory biologic scaffold (decellularized small intestinal submucosa) was treated with anti-inflammatory peptide amphiphiles (AIF-PAs) or control peptide amphiphiles and used for augmentation. Significant regenerative advantages of the AIF-PAs were observed including potent angiogenic responses, limited tissue collagen accumulation, and the modulation of macrophage and neutrophil responses in regenerated bladder tissue. Upon further characterization, a reduction in the levels of M2 macrophages was observed, but not in M1 macrophages in control groups, while treatment groups exhibited decreased levels of M1 macrophages and stabilized levels of M2 macrophages. Pro-inflammatory cytokine production was decreased while anti-inflammatory cytokines were up-regulated in treatment groups. This resulted in far fewer incidences of tissue granuloma and bladder stone formation. Finally, functional urinary bladder testing revealed greater bladder compliance and similar capacities in groups treated with AIF-PAs. Data demonstrate that AIF-PAs can alleviate galvanic innate immune responses and provide a highly conducive regenerative milieu that may be applicable in a variety of clinical settings.

1. Introduction

The cascade of events involved in the tissue regenerative process encompasses multiple innate biological programs that aid in regeneration. These are non-exclusively comprised of a robust inflammatory reaction at the onset of tissue damage subsequently accompanied by architectural and physiological remodeling of

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tissue. Although the inflammatory response initially acts as a protective mechanism to aid in repair and regeneration, impediments to tissue healing including perturbations in the inflammatory response, are attributable to further tissue damage and subvert proper tissue remodeling. The expected outcome of inflammation in a normal setting is to provide a suitable environment in order to recapitulate native tissue in form and in function through a series of interdependently choreographed events encompassing several distinct phases [1].

The inflammatory response to tissue injury is in part under the control of the innate immune system. Localized tissue injury induces the onset of leukocyte invasion, edema and pain of affected tissues [2]. The tissue infiltration of leukocytes via extravasation including macrophages, granulocytes (basophils, neutrophils, mast cells, and eosinophils) has been adequately demonstrated within the field of tissue regeneration as well as cancer initiation and progression [3,4]. Neutrophils and macrophages produce pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8 and TNFα. Neutrophils also possess the capacity to recruit more monocytes (and ultimately macrophages) thus continuing the cyclical process of tissue damage in a dysfunctional setting [5]. This acute inflammatory response can become chronic and eventually lead to tissue fibrosis. Abundantly fibrous tissue lacks proper physiological function as demonstrated in a number of different conditions including the formation of adhesions following surgery, idiopathic pulmonary fibrosis, and urinary bladder tissue regeneration [3,6,7]. In order to quell the inflammatory response and promote regenerative healing, the delivery of anti-inflammatory agents may prove beneficial in this setting.

Self-assembling peptide amphiphiles (PAs) have demonstrated utility in a wide range of settings and applications [8–10]. PAs are comprised of a hydrophobic alkyl segment attached to a peptide domain that includes a β-sheet forming segment. In aqueous environments, these molecules self-assemble through hydrophobic collapse of the alkyl domain in combination with hydrogen bonding in the β-sheet domain to produce high aspect-ratio nanofibers. PAs can be synthesized to present bioactive peptide epitopes on the nanofiber surface for recognition by cell receptors or for binding to other biomolecules in order to enhance biological function in vivo [8–10]. Specific epitopes that may be of interest in the context of tissue regeneration include anti-inflammatory sequences [11]. PAs presenting anti-inflammatory epitopes at high density could potentially be utilized to modulate inflammation-based reactions in a wide array of clinical settings. Within the context of this proof-of-concept study, we have attempted to specifically ascertain whether the application of PAs expressing anti-inflammatory peptides could 1) establish a comprehensive tissue regenerative milieu and 2) modulate components of the innate inflammatory response while utilizing a highly pro-inflammatory biological scaffold known to promote tissue fibrosis in a urinary bladder augmentation model [12,13].

2. Materials and methods

2.1. Anti-inflammatory peptide amphiphile synthesis

The anti-inflammatory peptide amphiphiles (AIF-PAs) were synthesized utilizing standard fluoroen-9-ylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) procedures as previously described [8]. Briefly, the AIF-PAs were synthesized from the C-terminus to N-terminus, with Rink Amide MBHA [4-(2′,4′-Dimethoxyphenyl-Fmoc-aminmethyl)-phenoxyacetamido-methylbenzhydryl amine resin] at the C terminus as the solid state support for the addition of Fmoc-protected AA. The Fmoc group was removed by agitating the resin in a solution of 30% piperidine in dimethylformamide (DMF) (v/v) for 10 min. This step was performed twice at the beginning of each AA coupling step. This was followed by a wash with dichloromethane (DCM), two washes with DMF, and finally two final washes in DCM. A Ninhydrin test was performed to confirm the presence of an N-terminus free amine indicated by a positive color change to purple. The AA coupling cocktail consisted of a 4× molar excess of the Fmoc AA, 3.95× molar excess of O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and a 6× molar excess of N,N-diisopropylethylamine (DIEA) all relative to the resin. The AA, HBTU, and DIEA were dissolved in approximately 20 mL of DMF. Once the Ninhydrin test confirmed the presence of free amino termini, the AA coupling cocktail was added to the resin and agitated for a minimum of 3 h. Following agitation, the resin was washed thoroughly with DMF 3× for approximately 1 min per wash and subsequently washed with DCM twice. A final Ninhydrin test was performed to confirm the AA had been coupled successfully indicated by a no color change. The remaining AA was added as described. Following the addition of the AA sequence, a palmitic acid (C16) tail was coupled for 2 h at 4× molar excess with 3.95× and 6× molar excess of HBTU and DIEA, respectively, dissolved in a 20:80 mixture of DCM:DMF.

To cleave the finished AIF-PA from the resin, a solution containing 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% nanopure water was mixed with the resin and agitated for at least 3 h. The solution was isolated from the resin into a 500 mL round bottom flask, and the resin was washed once with DCM. The TFA was removed using a rotary evaporator (R-210; Buchi Analytical Inc.) at 50 °C. Cold diethyl ether was added to remove soluble protecting groups remaining after cleaving and precipitate the AIF-PAs. The AIF-PA diethyl ether solution was centrifuged and decanted, leaving only the AIF-PA precipitate, which was then dried under vacuum for 2 days. Following cleavage, the AIF-PAs were purified via RP-HPLC (Varian Prostar; Varian Inc.) under either acidic (C-18 Atlantis Column; Waters Corp.) or basic (C-18 Gemini Column; Phenomenx Inc.) conditions based on the net charge of the complete AIF-PA at physiological pH. Purification was monitored by UV/Vis spectroscopy at 220 nm and 325 nm. Fractions of interest were confirmed to contain the target AIF-PA via mass spectrometry (6510 Q-TOF LC/MS 1200 Series; Agilent Technologies). Following purification, the AIF-PAs were subject to rotary evaporation before being lyophilized (FreeZone Plus 6; Labconco Corp.) for 3 days. Dried samples were stored at −20 °C until needed. The AA sequences (with the bioactive epitope in bold) of the AIF-PAs utilized throughout this study were: AIF-PA1: C_{16}-VVVAAAEEE**MQMKKVLDS**; AIF-PA2: C_{16}-VVVAAAEEE**HDMNKVLDL**; AIF-PA3: C_{16}-VVVAAAEEE**KVLDPVKG**; AIF-PA4: C_{16}-VVVAAAEEE**KVLDGQDP**; AIF-PA5: C_{16}-VVVAAAEEE**DPVKG**; AIFC-PA6 (Control): C_{16}-VVVAAAEEE**KLMSQKMVD**. AIFC-PA6 expressed a scrambled AA sequence that served as a control throughout experiments. Sequences were derived from studies of the uteroglobin protein [11,14]. All chemicals were purchased from Sigma–Aldrich Corporation.

2.2. Scanning electron microscopy of SIS scaffolds

Scanning electron microscopy (SEM) was performed using a Hitachi S4800-II scanning electron microscope (Hitachi Inc.) with a 5 kV accelerating voltage. To prepare samples for imaging, a sample of SIS coated with an AIF-PA was prepared by identical methods as were used for in vivo studies. An AIF-PA solution, induced to form a gel using a $CaCl_2$ solution, was coated onto one side of the surface of an SIS scaffold. The sample was fixed in 2% glutaraldehyde and 3% sucrose in phosphate buffered saline (PBS) for 30 min at 4 °C followed by sequential dehydration in an ascending series of ethanol. The scaffold was then dried at the critical point and coated with 8 nm OsO_4 prior to imaging. Images were collected of both the SIS surface alone and a representative sample of SIS with AIF-PA on the surface.

2.3. Rat bladder augmentation model

Athymic female rats (weighing ~200 g and 8–10 weeks old; National Cancer Institute Animal Production Program) underwent bladder augmentation as previously described [15]. Athymic rats were chosen for this study to solely examine the innate immune response to inflammation without influence of the adaptive immune response. In order to induce anesthesia, animals were given intraperitoneal injections of 60 mg/kg ketamine and 5 mg/kg xylazine. A second injection consisting of Buprenex (1 mg/kg) was administered subcutaneously to alleviate any pain/discomfort during and following surgical procedures. An approximate 1.5 cm midline vertical skin incision was created to expose the abdominal fascia and muscles. This was immediately followed by the separation of the wall leading to the identification of the bladder. An approximate 70% supratrigonal cystectomy was performed from anterior to posterior creating a clamshell. Immediately prior to bladder augmentation procedures, samples of the pro-inflammatory biological scaffold, small intestinal submucosa [SIS (0.5 × 0.5 cm); Cook Biotech.) [12,13,16] were thoroughly dip-coated separately in one of the previously described AIF-PAs following gelation procedures. The sections of SIS were dip-coated for approximately 20 s and allowed to adhere to the SIS for an additional 30 s. Dip-coating procedures were performed at room temperature in air. The cystectomized bladder defect was then augmented with: 1) SIS (non-AIF-PA coated SIS; $n = 8$ animals over both time-points); 2) AIF-PA1 coated SIS (denoted as SIS/AIF-PA1; $n = 11$ animals over both time-points); 3) AIF-PA2 coated SIS (denoted as SIS/AIF-PA2; $n = 8$ animals over both time-points); 4) AIF-PA3 coated SIS (denoted as SIS/AIF-PA3; $n = 3$); 5) AIF-PA4 coated SIS (denoted as SIS/AIF-PA4; $n = 3$ animals); 6) AIF-PA5 coated SIS (denoted as SIS/AIF-PA5; $n = 3$ animals); and 7) AIFC-PA6 coated SIS (denoted as SIS/AIFC-PA6; $n = 9$ animals over both time-points). AIF-PA3, AIF-PA4, and AIF-PA5 were not utilized for 5 week in vivo studies due to their lack of overall robustness with regard to various measurements taken at the 10 day time-point. The bladder was finally covered with omentum after being closed in a watertight manner utilizing 7-0 polyglactin suture. The abdominal wall was then