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The inhibition of platelet adhesion and activation on collagen during balloon angioplasty by collagen-binding peptidoglycans

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ABSTRACT

Collagen is a potent stimulator for platelet adhesion, activation, and thrombus formation, and provides a means for controlling blood loss due to injury, and recruiting inflammatory cells for fighting infection. Platelet activation is not desirable however, during balloon angioplasty/stent procedures in which balloon expansion inside an artery exposes collagen, initiating thrombosis, and inflammation. We have developed biomimetic polymers, termed peptidoglycans, composed of a dermatan sulfate backbone with covalently attached collagen-binding peptides. The peptidoglycan binds to collagen, effectively masking it from platelet activation. The lead peptidoglycan binds to collagen with high affinity (K_D = 24 nM) and inhibits platelet binding and activation on collagen in both static studies and under flow, while promoting endothelial regrowth on collagen. Application for angioplasty is demonstrated in the Ossabaw miniature pig by fast delivery to the vessel wall through a therapeutic infusion catheter with a proprietary PTFE porous balloon. The peptidoglycan is an approach for locally preventing platelet deposition and activation on collagen. It can be used during angioplasty to prevent platelet deposition on target vessels and could be used in any vessel, including those not amenable to stent deployment.

1. Introduction

An unintended consequence of angioplasty is damage to the vessel wall at the site of balloon deployment [1]. The layer of endothelial cells covering the internal lumen of the vessel is damaged, and the underlying collagenous connective tissue is exposed. Platelets are well adapted to bind to collagen where they become activated and release or upregulate numerous vasoactive agents and factors that induce coagulation and inflammation [2,3]. The body has evolved these responses as an effective means for controlling blood loss and fighting infection following injury; however, this same collagen-initiated coagulation and inflammatory response occurring inside the artery results in thrombosis and intimal hyperplasia [4–9].

Stents and drug-eluting stents are the current standard for angioplasty, although there are limitations of these devices and a need for a more robust treatment that can be used in all arteries. For example, stent placement in peripheral arteries is limited due to the flexible nature of these vessels and the resulting stresses that can lead to stent crushing or further vessel damage [10–13]. Branched, smaller diameter arteries, or vessels that have stents in place and require a second procedure are also difficult to address with current stent technologies. Drug-eluting balloons are one promising approach to address the limitation of stents [14,15]. These, like many of the drug-eluting stents, primarily employ the drugs Paclitaxel or Sirolimus [16], which are nonspecific and have cytotoxic or cytostatic effects. The drugs effectively prevent smooth muscle cell proliferation and intimal hyperplasia, but also prevent endothelial cells from regenerating to provide the necessary permanent cover to the underlying collagen. Consequently, underlying collagen remains exposed and this exposure has lead to the problem of late-stent thrombosis, particularly in high-risk acute coronary syndrome and in diabetes patients [17–19]. Accordingly, systemic dual antiplatelet therapy is the recommended treatment; though systemic compromise of platelets can cause bleeding complications that increase mortality risk [20–22].

Current approaches for improving the outcome of angioplasty target the downstream effects of inflammation [8,18]. For example, inflammation can be attenuated by systemically depleting monocytes at the time of angioplasty, resulting in inhibition of neointimal formation [23]. This approach requires systemic depletion of monocytes at the time of the invasive angioplasty procedure however, which could potentially carry new risks. Focusing on the initiating event, platelets are responsible for recruiting inflammatory cells to the balloon injured site through expression of p-selectin. The importance of platelets in thrombosis is well known, but less

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appreciated is their role in inflammation, which is fundamental for preventing intimal hyperplasia [23,24]. In a knockout mouse study, inhibition of neointimal formation could be achieved in the absence of platelet p-selectin, not endothelial p-selectin, thus demonstrating the importance of platelets in the adverse inflammation response at the vessel wall [25].

We have targeted the initial platelet binding event on balloon injured vessels by locally masking exposed collagen in the vessel wall following angioplasty. We have developed bioinspired materials termed peptidoglycans, which are composed of a polysaccharide backbone with covalently attached collagen-binding peptides [26,27]. The peptidoglycan binds to collagen through the physical peptide–collagen interactions, and when bound to collagen the peptidoglycan acts as a physical barrier to platelet attachment and subsequent activation as shown in Fig. 1. The peptide sequence is derived from a platelet receptor to collagen, and as such the peptidoglycan directly competes for platelet binding while masking the collagen surface with a hydrophilic barrier [28,29]. Here we demonstrate *in vitro* efficacy of the peptidoglycan, and a clinically relevant method for delivery during angioplasty.

2. Methods

2.1. Reagents

Peptide RRANAALKAGELYKSILYGC (SILY) and a biotin labeled version of the peptide ($SILY_{biotin}$) were purchased from Genscript (Piscataway, NJ). Dermatan sulfate (DS, $MW_{avg} = 46275$ Da) was purchased from Celsus Laboratories (Cincinnati, OH). Sodium meta-periodate and crosslinker BMPH were purchased from Thermo Fisher Scientific (Waltham, MA). Fibrillar equine collagen was purchased from Chronolog (Havertown, PA). Local infusion catheters with a proprietary PTFE porous delivery balloons (ClearWay™ Rx) were kindly provided by Atrium Medical (Hudson, NH). All other reagents and supplies were purchased from VWR (West Chester, PA) unless otherwise stated.

2.2. Peptidoglycan synthesis

The peptidoglycan was synthesized as previously described with modifications [26]. DS was oxidized by standard periodate oxidation following manufacturers protocol, in which the degree of oxidation was controlled by varying amounts of sodium meta-periodate. Oxidized DS was then coupled to the heterobifunctional crosslinker BMPH forming DS-BMPH. Finally, SILY was coupled to DS-BMPH through its terminal cysteine residue forming the final product DS-SILY. Purifications were performed at each step by size exclusion chromatography, and the number of attached peptides was determined by the consumption of BMPH in the second reaction step. The final product $DS\text{-}SILY_n$ in which n indicates the number of attached SILY peptides was purified in ultrapure water, lyophilized and stored at −20 °C until further testing. A biotin labeled version of the peptidoglycan was also synthesized by reacting 2 mol of $SILY_{biotin}$ per mole of DS-BMPH for 1 h, followed by addition of unlabeled SILY to complete the reaction and form $DS\text{-}SILY_{n\text{-}biotin}$.

2.3. Peptidoglycan binding to collagen

Fibrillar collagen was coated onto the surface of a 96-well high bind plate (Greiner, Monroe, NC) at a concentration of 50 μg/mL diluted in isotonic glucose. Plates were incubated overnight at 4 °C. Unbound collagen was removed by rinsing 3 times with 1× PBS pH 7.4. Plates were then blocked with 1% BSA for 3 h at room temperature. For binding affinity calculation, $DS\text{-}SILY_{biotin}$ was dissolved at varying concentrations in 1× PBS pH 7.4 containing 1% BSA and was immediately added to the collagen surfaces, and allowed to incubate for 15 min at room temperature. Plates were then rinsed 3 times with 1× PBS pH 7.4 containing 1% BSA. $DS\text{-}SILY_{n\text{-}biotin}$ was detected using streptavidin-HRP (R&D Systems, Minneapolis, MN). The binding affinity was determined by fitting the saturation binding curve and calculating the inflection point. The peptidoglycan $DS\text{-}SILY_{18}$ which bound with highest affinity while maintaining solubility was then used for all successive experiments.

To determine the diffusion of collagen-bound peptidoglycan over time, peptidoglycan $DS\text{-}SILY_{18\text{-}biotin}$ was incubated at a concentration of 10 μM as in affinity studies. Plates were incubated at 37 °C with orbital rotation at 300 rpm and wells were rinsed 3 times daily throughout the experiment. Rinsing conditions were 200 μL/well of 1× PBS pH 7.4 repeated 3 times at each time point. The amount of bound $DS\text{-}SILY_{18\text{-}biotin}$ was detected by the same methods for affinity testing at varying time points up to 11 days.

2.4. Ex-vivo visualization of bound peptidoglycan

Carotid arteries were harvested from Yorkshire pigs immediately after necropsy from separate studies. Arteries were immediately cut open and rinsed with 1× PBS pH 7.4. The internal lumen was then gently denuded of the endothelial layer with a rubber policeman, rinsed with 1× PBS and cut into 4 mm^2 segments and placed into a 96-well plate. Denuded artery segments were incubated for 15 min with 100 μL of 10 μM peptidoglycan $DS\text{-}SILY_{18\text{-}biotin}$ at room temperature followed by extensive rinsing with 1× PBS pH 7.4. Control arteries were treated identically but were not denuded. Arteries were then snap frozen in liquid nitrogen, cut into 7 μm

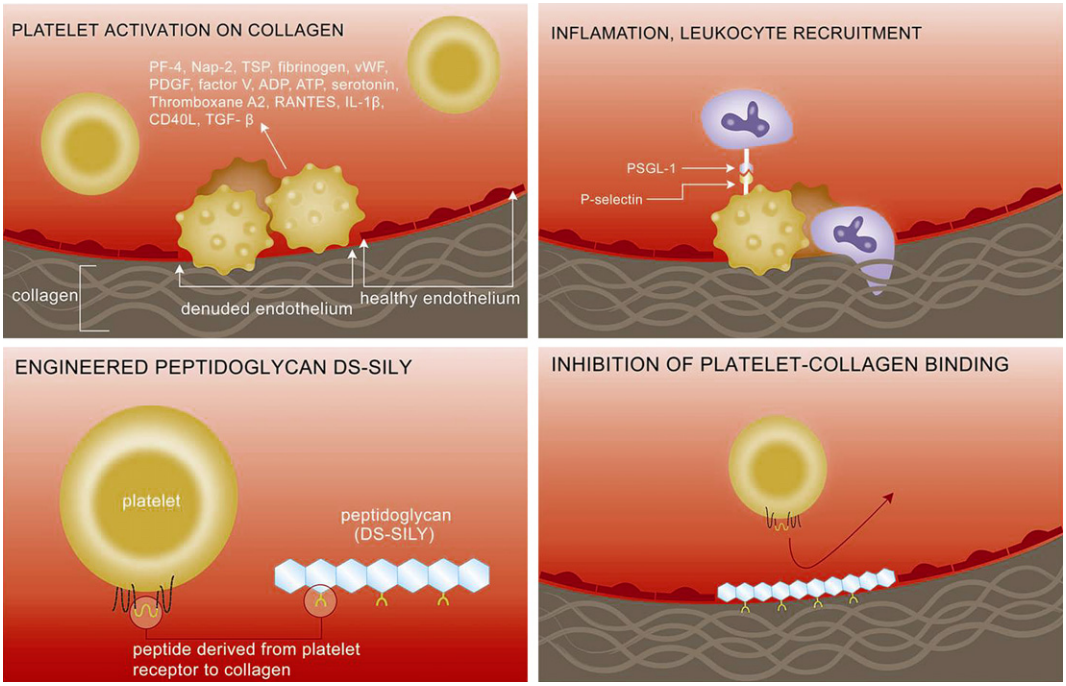

Fig. 1. Masking of collagen by bioinspired peptidoglycan. Platelet binding and activation on denuded arteries initiates coagulation and inflammation. We have developed a bioinspired mask, termed peptidoglycan, which inhibits platelet binding and activation on exposed collagen in denuded arteries.