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Minocycline enhances the mesenchymal stromal/stem cell pro-healing phenotype in triple antimicrobial-loaded hydrogels

Alberto Daniel Guerra [a], Warren E. Rose [a], Peiman Hematti [b], W. John Kao [a,c,d,*,1]

[a] School of Pharmacy, Division of Pharmaceutical Sciences, Pharmacy Practice Division, University of Wisconsin-Madison, 777 Highland Avenue, Madison, WI 53705, USA
[b] School of Medicine and Public Health, Department of Medicine, Wisconsin Carbone Cancer Center, University of Wisconsin-Madison, 1685 Highland Avenue, Madison, WI 53705, USA
[c] College of Engineering, Department of Biomedical Engineering, University of Wisconsin-Madison, 1415 Engineering Drive, Madison, WI 53706, USA
[d] School of Medicine and Public Health, Department of Surgery, University of Wisconsin-Madison, 1685 Highland Avenue, Madison, WI 53705, USA

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) have demonstrated pro-healing properties including an anti-inflammatory cytokine profile and the promotion of angiogenesis via expression of growth factors in pre-clinical models. MSCs encapsulated in poly(ethylene glycol) diacrylate (PEGdA) and thiolated gelatin poly(ethylene glycol) (Gel-PEG-Cys) crosslinked hydrogels have led to controlled cellular presentation at wound sites with favorable wound healing outcomes. However, the therapeutic potential of MSC-loaded hydrogels may be limited by non-specific protein adsorption on the delivery matrix that could facilitate the initial adhesion of microorganisms and subsequent virulent biofilm formation. Antimicrobials loaded concurrently in the hydrogels with MSCs could reduce microbial bioburden and promote healing, but the antimicrobial effect on the MSC wound healing capacity and the antibacterial efficacy of the hydrogels is unknown. We demonstrate that minocycline specifically induces a favorable change in MSC migration capacity, proliferation, gene expression, extracellular matrix (ECM) attachment, and adhesion molecule and growth factor release with subsequent increased angiogenesis. We then demonstrate that hydrogels loaded with MSCs, minocycline, vancomycin, and linezolid can significantly decrease bacterial bioburden. Our study suggests that minocycline can serve as a dual mechanism for the regenerative capacity of MSCs and the reduction of bioburden in triple antimicrobial-loaded hydrogels.

Statement of Significance

Wound healing is a complex biological process that can be hindered by bacterial infection, excessive inflammation, and inadequate microvasculature. In this study, we develop a new formulation of poly (ethylene glycol) diacrylate and thiolated gelatin poly(ethylene glycol) crosslinked hydrogels loaded with minocycline, vancomycin, linezolid, and mesenchymal stromal/stem cells that induces a favorable wound healing phenotype in mesenchymal stromal/stem cells and prevents bacterial bioburden on the hydrogel. This combinatorial approach to biomaterial development has the potential to impact wound healing for contaminated full thickness cutaneous wounds.

1. Introduction

Bone marrow-derived mesenchymal stromal/stem cells (MSCs) have demonstrated pro-healing properties through multiple mechanisms: an anti-inflammatory cytokine expression profile, decreased fibrotic granular tissue, enhanced keratinocyte proliferation, inhibited myofibroblast differentiation, and elevated proliferation of microvascular endothelial cells with subsequent angiogenesis [1–8]. The wound healing properties of MSCs are maintained when MSCs are encapsulated in a poly(ethylene glycol)-diacrylate (PEGdA) and thiolated gelatin poly(ethylene glycol) (Gel-PEG-Cys) crosslinked hydrogel utilized for spatially and temporally controlled cellular presentation and the promotion of full-thickness cutaneous wound healing [9–12]. The hydrogel utilizes a modified gelatin with cysteine through a PEG linker that leads to cytoplasmic spreading and the formation of cellular

* Corresponding author at: 777 Highland Avenue, 7123 Rennebohm Hall, Madison, WI 53705, USA.
E-mail addresses: aguerra@wisc.edu (A.D. Guerra), warren.rose@wisc.edu (W.E. Rose), pxh@medicine.wisc.edu (P. Hematti), WJKao@wisc.edu (W.J. Kao).
[1] Current address: 10/F Knowles Building, Pokfulam, Hong Kong.

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networks that improves cellular delivery and extends survival time of MSCs with increased residence time at the wound site compared to an intravenous injection [9,10,12]. However, the therapeutic potential of biomaterial cell-based therapies may be limited by non-specific protein adsorption on the delivery matrix similar to that of implants. This in turn could facilitate the initial adhesion and replication of wound-associated microorganisms such as *Staphylococcus aureus* (SA), and a quorum sensing response to high cell density that leads to SA attachment, virulence expression and production, and eventual biofilm formation [13–17]. SA within biofilms produce extracellular polymer substance that results in bacterial resistance to immune cell recognition and elimination as well as reduced antibacterial activity [14]. Biofilm bacteria remain virulent by creating an acidic and hypoxic microenvironment that contributes to primary immune cell death and tissue necrosis. Sloughing of bacteria from the implant surface results in a dysfunctional innate immune response through high secretion of proteases, reactive oxygen species, and pro-inflammatory cytokines at the implant site [18,19]. Virulent SA that form biofilms is one of the most common pathogens in non-healing wounds. Over half of patient chronic wound samples have shown the presence of biofilms, and SA has been used in infected rat wound models [20–22]. Opportunely, the hydrogel system can be loaded with multiple prophylactic antimicrobials with controlled release kinetics [23–25]. We have previously demonstrated that hydrogels loaded concurrently with MSCs and minocycline significantly lowers SA colony forming potential in solution while maintaining MSC viability and multipotency [26]. However, at long culture times (16 h), planktonic SA growth was observed that could lead to enhanced bioburden [18,19,26,27]. The addition of other broad-spectrum antimicrobials to the hydrogel system to prevent bacterial growth through multiple mechanisms may lead to increased antimicrobial efficacy. However, the pharmacological effects of antimicrobials on the MSC wound healing profile and the bacterial growth inhibition of MSC and triple antimicrobial-loaded hydrogels has not been investigated. In this study, we develop hydrogels loaded with MSCs and an antimicrobial combination that includes minocycline, vancomycin, and linezolid and investigate the mechanistic insights of the pro-healing outcome by quantifying the antimicrobial-induced effects on MSC migration capacity, proliferation, gene expression, extracellular matrix (ECM) attachment, cytokine, adhesion molecule, and growth factor production with subsequent angiogenesis potential. We then determine the release kinetics of the antimicrobials from the MSC-loaded hydrogels and then determine the antibacterial capacity of MSC and triple antibiotic-loaded hydrogels against SA.

2. Materials and methods

2.1. Cell isolation and culture

2.1.1. MSC isolation, characterization and culture

MSCs were isolated from discarded filters of bone marrow harvests of healthy adult human donors based on a protocol approved by the University of Wisconsin Hospital and Clinics Regulatory Committee per our published protocols [10,28]. Isolated MSCs at passage 4 were characterized for positive and negative markers via flow cytometry, and for multidifferentiation potential as previously described [10,28,29]. MSCs were cultured in 75 cm^2 tissue culture flasks (TPP, St. Louis, MO) with Dulbecco's Modified Eagle Medium (DMEM, Cellgro Mediatech, Inc., Corning, NY), 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2 mM non-essential amino acids (NEAA) with medium changes every 3–5 days. Only MSCs passage 4–8 were used in this study.

2.1.2. S. aureus culture

SA (ATCC strain #29213, Manassas, VA) culture vials stored at −80 °C were thawed, plated on trypticase soy agar (TSA, Becton Dickinson, Sparks, MD) Petri dishes (Fischer Scientific) and cultured as previously described [26]. The SA culture was allowed to proliferate in suspension on a shaker at 170 rpm at 37 °C until desired growth phase optical density was achieved (OD_{600} = 0.1, 1×10^4 CFU/mL) as measured by the spectrophotometer (Nanodrop 2000x UV–vis Spectrophotometer, Thermo Scientific). SA at OD_{600} = 0.1 represents the pre-log growth phase (lag phase) in which bacteria will have to replicate at the wound site to establish a clinical infection, representing a wound infection model for most clinical cases [30,31].

2.1.3. HUVEC culture

Human umbilical vein endothelial cells (HUVECs) (ThermoFisher Scientific) were thawed and cultured in 75 cm^2 tissue culture flasks (TPP) with medium 200 (ThermoFisher Scientific) with 10% low serum growth supplement (ThermoFisher Scientific) with medium changes every 3–5 days. Only passage 4–8 HUVECs were used in this study.

2.2. Development of MSC and antimicrobial-loaded hydrogels

2.2.1. Toxicity of MSCs Exposed to drug cocktails

MSCs were plated at 40,000 cells/mL in 96-well TCPS plates (BD Biosciences). A 5 mg/mL stock of minocycline (Research Products International, Mt. Prospect, IL), vancomycin (Dot Scientific Inc., Burton, MI), and linezolid (AK Scientific Inc., Union City, CA) were made in MSC growth medium and were diluted to appropriate working solutions. The appropriate amount of each working solution was added to make the following drug conditions of minocycline + vancomycin + linezolid in μg/mL: 25/50/5, 35/30/7.5, 50/40/10, 75/60/15, and 100/80/20, respectively, determined from independent preliminary MSC toxicity studies of each antimicrobial. Plates were incubated for 1, 3, 5, and 7 days. Respective media was changed after 3 days in the 5 and 7 day treatment groups to avoid cell death as a result of a lack of nutrients and a buildup of toxic metabolism by-products in the medium. After respective treatment times, the medium was removed from the well and a LIVE/DEAD® (Invitrogen) stain was applied with subsequent imaging and fluorescence quantification followed by a CellTiter-Blue® (Promega, Madison, WI) stain with subsequent quantification as previously described [26].

2.2.2. Hydrogel preparation

Hydrogel formulations were made in PBS to make a 0.5% (w/v) Irgacure 2959 photoinitiator (BASF, Ludwigshafen, Germany), 10% (w/v) PEGdA and 10% (w/v) Gel-PEG-Cys sterile-filtered solution prepared and characterized as described previously [32,33]. A MSC suspension was added into the formulation at 1×10^6 cells/mL. For hydrogel samples loaded with antimicrobials, 1 mg/mL stocks were made in PBS and the appropriate amount was added to a solution containing photoinitiator, PEGdA, and Gel-PEG-Cys in PBS. The antimicrobial load formulation for preliminary MSC screening was selected based on the toxicity screening stated above. Hydrogels loaded concurrently with MSC and antimicrobials individually or in combination were prepared following similar procedures as described above. The formulations were then pipetted (100 μL) into a 10 mm glass-bottom Petri dish (In Vitro Scientific, Sunnyvale, CA) with a circular recess and then polymerized by exposing to UV light (λ_{max} = 365 nm, 100 W/cm^2) for 2 min. MSC medium (2 mL) with corresponding drug concentrations (when applicable) was added to the tissue culture Petri dish to