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Effects of doxycycline on mesenchymal stem cell chondrogenesis and cartilage repair

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SUMMARY

Objective: Strategies to improve cartilage repair tissue quality after bone marrow cell-based procedures may reduce later development of osteoarthritis. Doxycycline is inexpensive, well-tolerated, and has been shown to reduce matrix-metalloproteinases (MMPs) and osteoarthritis progression. This study tests the hypotheses that doxycycline reduces MMP, enhances chondrogenesis of human bone marrow-derived mesenchymal stem cells (hMSC), and improves *in vivo* cartilage repair.
Design: Ninety hMSC pellets were cultured in chondrogenic media with either 0-, 1- or 2-μg/mL doxycycline. Pellets were evaluated with stereomicroscopy, proteoglycan assay, qRT-PCR, and histology. Osteochondral defects (OCDs) were created in the trochlear grooves of 24-Sprague-Dawley rats treated with/without oral doxycycline. Rats were sacrificed at 12-weeks and repair tissues were examined grossly and histologically.
Results: hMSC pellets with 1-μg/mL ($P = 0.014$) and 2-μg/mL ($P = 0.002$) doxycycline had larger areas than pellets without doxycycline. hMSC pellets with 2-μg/mL doxycycline showed reduced *mmp*-13 mRNA ($P = 0.010$) and protein at 21-days. Proteoglycan, DNA contents, and mRNA expressions of chondrogenic genes were similar ($P > 0.05$). For the *in vivo* study, while the histological scores were similar between the two groups ($P = 0.116$), the gross scores of the OCD repair tissues in doxycycline-treated rats were higher at 12-weeks ($P = 0.017$), reflective of improved repair quality. The doxycycline-treated repairs also showed lower MMP-13 protein ($P = 0.029$).
Conclusions: This study shows that doxycycline improves hMSC chondrogenesis and decreases MMP-13 in pellet cultures and within rat OCDs. Doxycycline exerted no negative effect on multiple measures of chondrogenesis and cartilage repair. These data support potential use of doxycycline to improve cartilage repair to delay the onset of osteoarthritis.

Introduction

Osteoarthritis is a leading cause of disability. Focal cartilage injuries are known to accelerate the degeneration of surrounding cartilage and to hasten the onset of osteoarthritis. Articular cartilage has limited capacity to repair following injury[1,2]. Microfracture (bone marrow stimulation) is commonly used to treat focal cartilage injuries. This technique involves the recruitment of bone marrow-derived mesenchymal stem cells (MSCs) to the defect area to participate in cartilage repair. However, the resulting repair does not restore hyaline articular cartilage, but instead results in formation of a mechanically inferior fibrocartilaginous scar tissue[1,2]. Therefore, clinical strategies are needed to improve the type and quality of repair tissue following marrow stimulation procedures to potentially delay or prevent the onset of osteoarthritis.

Doxycycline is a widely available, inexpensive and well-tolerated antibiotic that has a long clinical history in humans. In addition, doxycycline has been effectively used with the tetracycline-inducible gene regulation system for controlled gene therapy applications in the articular joint as well as other organ systems across multiple animal models[3,4]. Besides anti-microbial and transgene-inducing functions, doxycycline may also have intrinsic benefits to articular cartilage. Prior studies have shown that the tetracycline-class of medications has the ability to inhibit matrix-metalloproteinases (MMPs), with doxycycline being the most studied for this purpose[5–10].

MMPs are a family of zinc-dependent proteinases that differ slightly in substrate specificity. They function in maintaining the

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homeostasis of extracellular matrix (ECM) by degrading ECM molecules, such as collagens and proteoglycans[11]. Down-regulation of MMP increases the amount of collagens retained in the ECM[9]. MMP is upregulated in arthritis and following cartilage injury[6,9,10,12,13]. Among the MMP collagenases, MMP-1 (collagenase-1), -8 (collagenase-2), and -13 (collagenase-3) cleave interstitial collagens[10,14]. MMP-13 is the most active for degradation of collagen-II, and has therefore been implicated in arthritic disease processes[15,16]. In transgenic mice models, overexpression of MMP-13 has induced osteoarthritic changes[16] while the absence of MMP-13 inhibited cartilage erosion[17].

Oral administration of doxycycline has been postulated to have beneficial effects on articular cartilage by inhibiting MMP, specifically MMP-1, -3 (stromelysin), and -13[5–10,18]. Doxycycline administration *in vivo* has been shown to decrease MMP levels in small animal models, as well as in human osteoarthritic samples[19,20]. When doxycycline was administrated prophylactically in animal models, decreased MMP activity was correlated to reduced proteoglycan loss from cartilage ECM, as well as to reduced degenerative changes in the weight-bearing areas of articular cartilage[21,22]. In a randomized, double-blinded, placebo-controlled study evaluating the effects of doxycycline on the progression of joint space narrowing in mildly arthritic knees, the doxycycline treatment group had a decreased rate of narrowing by 40% at 16-months and 33% at 30-months as compared to placebo ($P = 0.017$)[6]. On the other hand, other animal studies have not demonstrated any effect of doxycycline treatment on joint disease and/or MMP activities[23,24]. Hence numerous studies exist on the effect of doxycycline on cartilage degeneration, however with conflicting results.

Fewer studies have evaluated the effect of doxycycline on musculoskeletal repair processes. In a study using a rat laminectomy defect model, rats that received intra-peritoneal doxycycline showed enhanced wound healing, potentially due to reduction of scar tissue formation[25]. In another study, oral doxycycline was shown to inhibit MMP and improve and the strength of rotator cuff repairs in rats[19]. This study found that the doxycycline administered group had improved load to failure as well as greater collagen organization compared to controls and correlated these findings to a significant decrease in MMP-13 activity. In contrast, another study investigating the healing of Achilles tendon in rats showed decreased strength of the repaired tendon in the doxycycline group[26]. While the effects of doxycycline on musculoskeletal tissue repair processes may be variable, its effect on cartilage repair with MSCs as well as on MSC chondrogenesis is largely unknown. This study was performed to test the hypotheses that doxycycline reduces MMP expression, enhances human mesenchymal stem cell (hMSC) chondrogenesis, and improves *in vivo* cartilage repair.

Methods

In vitro studies

hMSC chondrogenic differentiation

Ninety pellet cultures were prepared using human MSCs from a single donor cultured to passage 3 according to previously described procedures[27]. Briefly, 2.5×10^5 hMSCs were pelleted by centrifugation at $500\times g$ for 15-min. The resulting pellets were cultured in 0.5-mL of pre-defined chondrogenic medium containing high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco), with 1% penicillin-streptomycin (Gibco), 10^{-7}-M dexamethasone (Sigma–Aldrich), 50-μg/mL L-ascorbic acid-2-phosphate (Sigma–Aldrich), 40-μg/mL proline (MP Biomedicals, Solon, USA), and 1% BD™ ITS + Premix (Becton–Dickinson), 10-ng/mL transforming growth factor-beta 1 (TGF-β_1, R&D Systems). The chondrogenic medium was supplemented with 0-, 1-, or 2-μg/mL of doxycycline: 30 pellets per each of doxycycline concentrations. The pellets were incubated at 37°C in 5% CO_2 for 3-, 7-, 14-, or 21-days, and the media was refreshed every 2–3 days.

Chondrogenic hMSC pellet assessments

Pellet area and histological evaluation

At 14- and 21-days of culture in chondrogenic media supplemented with 0-, 1-, or 2-μg/mL doxycycline, three hMSC chondrogenic pellets per group were analyzed grossly and for cross-sectional areas. Macroscopic assessment was performed using stereomicroscopy (MVX-10 MacroView Systems, Olympus) equipped with a DP71 camera (Olympus). The pellet area was measured using DP2-BSW software (Olympus). The pellets were subsequently fixed in 10% formalin and embedded in paraffin for histological analyses. Five-μm cross-sections were stained with 0.1% Safranin-O/0.5% Fast green for sulfated glycosaminoglycan (GAG) and alizarin-red for calcium[28], and immunostained for MMP-13 according to standard protocols. Briefly for MMP-13 immunohistochemistry (IHC), sections were de-paraffined and rehydrated via conventional methods. Antigen retrieval was performed by heating and cooling the slides in 10-mM sodium citrate (pH 6.0) with 0.05% Tween-20 buffer (Sigma–Aldrich) for 20-min each at 85°C and room temperature. The sections were then digested in 1-mg/mL hyaluronidase in 0.1-M sodium acetate buffer (both from Sigma–Aldrich) for 30-min in 37°C. The sections were then blocked with 10% goat serum (Vector Laboratories) and 1% bovine serum albumin (BSA, Fisher Scientific) for 1-h. The sections were incubated with a rabbit polyclonal antibody against MMP-13 (Abcam) at 1:100 dilution in 1% BSA overnight at 4°C. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide (Sigma) in tris-buffered saline (TBS, Calbiochem) for 15-min at room temperature. The slides were then treated with secondary goat polyclonal anti-rabbit antibody (Abcam) in 1% BSA in 1:100 dilution for an hour, then developed using ImmPACT™ AEC Peroxidase Substrate (Vector Laboratories), according to the manufacturer's instructions. The slides were counterstained with hematoxylin (Vector Laboratories). Images of all the stained sections were captured using TE-2000U Eclipse microscope (Nikon) equipped with a DP71 camera. The MMP-13 IHC images in Fig. 4 had the contrast increased by the

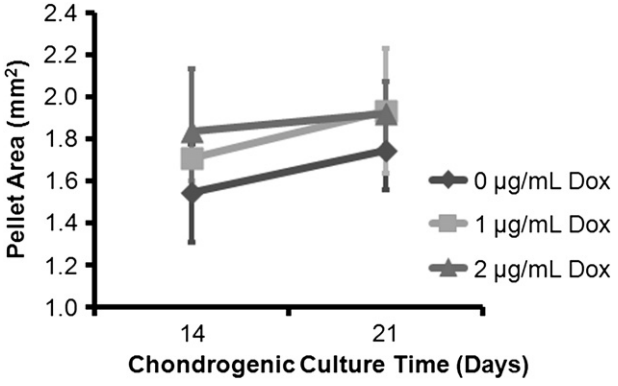

Fig. 1. Doxycycline increased MSC pellet area
Cross-sectional areas were measured from the hMSC pellets cultured in chondrogenic media with 0-, 1-, or 2-μg/mL Dox for 14- and 21-days. Pellet area increased from 14- to 21-days in chondrogenic culture period ($P = 0.002$). As well, the pellet area was significantly affected by the doxycycline concentration in the chondrogenic media regardless of the timepoints ($P = 0.002$): 0-μg/mL Dox pellets were smaller than the 1-μg/mL ($P = 0.014$) and 2-μg/mL Dox pellets ($P = 0.002$). There was no difference in sizes between the 1- and 2-μg/mL Dox pellets ($P = 0.535$). $n = 3$/group.