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In vivo reduction or blockade of interleukin-1β in primary osteoarthritis influences expression of mediators implicated in pathogenesis

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SUMMARY

Objective: Diminish interleukin-1β (IL-1β) signaling in a model of primary osteoarthritis by RNA interference-based transcript reduction or receptor blockade, and quantify changes incurred on transcript expression of additional mediators.

Methods: Knees of Hartley guinea pigs were collected at 120 and 180 days of age following injection with viral vectors ($N = 4$/treatment group/date) at 60 days. Two groups received either adeno-associated viral serotype 5 vector containing a knockdown sequence (TV), or adenoviral vector encoding for IL-1 receptor antagonist protein (Ad-IRAP); treatments were contrasted with opposite knees administered corresponding vector controls. A third group evaluated TV relative to saline-only injected knees. Chondropathy and immunohistochemistry findings were compared to untreated guinea pigs. Transcript expression levels in cartilage were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method and analyzed by one-way analysis of variance (ANOVA) with pairwise comparisons using Tukey 95% confidence intervals.

Results: Vector transduction was confirmed at both harvest dates. TV and Ad-IRAP, relative to vector controls, significantly decreased IL-1β. Inflammatory mediators [tumor necrosis factor-α (TNF-α), IL-8, interferon-γ (IFN-γ)], and catabolic matrix metalloproteinase 13 (MMP13) were also decreased, while anabolic transforming growth factor-β1 (TGF-β1) was increased. IL-1β was also decreased by TV vs saline, with a decrease in MMP13 and increase TGF-β1; TNF-α, IL-8, and IFN-γ were transiently increased.

Conclusions: This work confirmed that a reduction in IL-1β signaling was accomplished by either method, resulting in decreased expression of three inflammatory mediators and one catabolic agent, and increased expression of an anabolic molecule. Thus, evidence is provided that IL-1β serves a role *in vivo* in spontaneous osteoarthritis and that these translational tools may provide beneficial disease modification.

Introduction

Osteoarthritis (OA) is the leading cause of physical disability in developed nations[1]. Unfortunately, multiple molecular and biomechanical factors contribute to the pathogenesis of this degenerative process and restorative treatment options continue to be elusive[2]. Interleukin-1β (IL-1β), a pro-inflammatory cytokine that stimulates joint tissue to produce several proteases involved in cartilage degradation, is implicated as a principal instigator of OA[3]. Inhibiting the biological activities of IL-1β through RNA interference (RNAi) or receptor antagonism may delineate the contribution of its signaling pathway to disease and offers promise as a translational therapeutic strategy. Efforts to reduce or block the effective concentration of IL-1β have been demonstrated using diacerein, a compound that inhibits IL-1β production from synovium and cartilage[4–10], or IL-1 receptor antagonist protein (IRAP) in experimental OA[11–15]. Although these studies suggest that IL-1β is a viable target to modify development and succession of secondary joint deterioration, the majority of human cases of OA are idiopathic[16] and work is warranted to evaluate the definitive role of IL-1β in the context of spontaneous disease[3,17].

Recently, our laboratory provided a comprehensive immunohistochemical map describing the temporal expression and tissue distribution of IL-1β through progression of OA in two strains of guinea pigs with varying propensity for spontaneous knee joint disease[18]. At 60 days of age, IL-1β was detected in cartilage, menisci, synovium, and subchondral bone in both strains. Persistent

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expression was found in these tissues in OA-prone Hartley animals at 120 and 180 days, while OA-resistant Strain 13 animals demonstrated a significant reduction in immunostaining. Enduring IL-1β levels in Hartley animals appeared to coincide with histologic onset of OA, and this aberrant expression may correlate to early incidence of disease. Although findings suggested, but did not confirm, a cause-and-effect relationship between IL-1β expression and OA, we demonstrated a window when targeted cytokine reduction and/or blockade may identify such a connection and point toward mechanistic components. As such, the primary aim of this study was to minimize IL-1β-mediated signaling *via* RNAi-based transcript reduction or receptor blockade and quantify changes incurred on expression of mediators implicated in pathogenesis. We accomplished these aims *via* independent administration of a validated adeno-associated viral serotype 5 vector (AAV5) containing a short hairpin (sh)RNA knockdown sequence[19], or an adenoviral (Ad) vector encoding for recombinant human IRAP (hIRAP). These treatments were contrasted with opposite knees receiving corresponding vector controls with the goal of defining the contribution of IL-1β to spontaneous OA. A secondary aim was to challenge the efficacy and potential therapeutic viability of the AAV5 vector relative to saline-only injected control knees. Histologic chondropathy and immunohistochemistry (IHC) findings were compared to untreated guinea pigs.

Materials & methods

This study was performed according to the Guide for the Care and Use of Laboratory Animals of the NIH; all procedures were approved by the Institutional Laboratory Animal Care and Use Committee at the host university. Male Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were utilized in this study ($N = 32$) starting at 60 days of age. Animals were housed individually in solid bottom cages and allowed *ad libitum* water and guinea pig chow (Harlan Teklad 7006) until euthanasia. Body weight (grams) at time of harvest was recorded.

Experimental design

AAV5 vectors allowed simultaneous expression of shRNA *via* the U6 promoter and cytomegalovirus (CMV)-driven enhanced green fluorescent protein (GFP) expression. Plasmids containing the IL-1β specific shRNA knockdown sequence or non-targeting shRNA control sequence (Table IA) were used to produce targeting knockdown vector (TV) and non-targeting control vector (NTV), respectively, by the Viral Vector Core at Nationwide Children's Hospital[19,20]. Ad vectors contained coding regions for CMV-driven firefly luciferase (Ad-Luc) or hIRAP (Ad-hIRAP) and were propagated, as described[12]. Active protein production from Ad vectors was confirmed *in vitro* using bioluminescent luciferase detection or ELISA analyses for hIRAP (R&D Systems, Minneapolis, MN); results were positive for intended proteins, only.

Animals were injected at 60 days of age (four treatment groups; four animals/treatment group per collection date) and harvested at either 120 or 180 days of age. Vectors were aseptically administered into the medial aspect of the knee just distal to the femoral condyle using a 1/2cc 28g insulin syringe (Becton Dickinson, Franklin Lakes, NJ). Animals were assigned to one of two groups to investigate reduction/blockade of IL-1β vs respective vector controls: TV or NTV (1×10^{12} DNase resistant particles) were injected into opposite knees (group 1); Ad-Luc or Ad-hIRAP (2×10^{11} infectious units (IFUs)) were injected into opposite knees (group 2). Group 3 animals received TV or an equivalent volume of phosphate buffered saline (PBS) in opposite knees to challenge the efficacy of TV in the absence of vector control. Group 4 animals did not receive injections in either knee to allow reference of the above treatment groups to untreated controls. A total volume of 100 μl was administered; dosages and final injection volume were consistent with peer-reviewed manuscripts[19,20].

For groups 1–3, RNA was extracted from weight-bearing cartilage taken from the lateral femoral condyle and lateral tibial plateau of opposite knees for gene expression analyses. DNA was collected from patellar cartilage to quantify viral particle numbers. The medial femoral condyle and medial tibial plateau were processed for IHC and histology. Whole knee joints from group 4 animals were processed for IHC and histology.

Table I
A. Targeting knockdown and control shRNA sequences

Targeting knockdown IL-1β sequence	GCCAGGATATAATTGACTTCACGAATGAAGTCAATTATATCCTGGC
Non-targeting control sequence	GGATATATCCCGAACTAGACACGAATGTCTAGTTCGGGATATATCC

B. Specific primers used for real-time qRT-PCR

Transcript of interest	Primer sequences (5′–3′)
18S ribosomal RNA	F: TGCATGGCCGTTCTTAGTTG
	R: AGTTAGCATGCCAGAGTCTCGTT
GAPDH	F: GTATCGTGGAAGGACTCATGACC
	R: GTTGAAGTCACAGGACACAACCT
GFP	F: CATGATATAGACGTTGTGGCTGTTG
	R: AAGCTGACCCTGAAGTTCATCTGC
Firefly luciferase*	F: GCCTGAAGTCTCTGATTAAGT
	R: ACACCTGCGTCGAAGT
CVM promoter	F: GGTCTATATAAGCAGAGCTG
	R: GTGGTATGGCTGATTATGATCAG
IL-1β	F: ACGCCTGGTGTTGTCTGAC
	R: GGGAACTGAGCGGATTC
IL-8	F: GGCAGCCTTCCTGCTCTCT
	R: CAGCTCCGAGACCAACTTTGT
Human IRAP†	F: TGGCTAACTAGAGAACCCACTGCT
	R: TTCTGAAGGCTTGCATCTTGCTGG
Collagenase 3 (MMP13)	F: TTCTGGCACATGCTTTTCCTC
	R: GGTTGGGGTCTTCATCTCCTG
TGF-β1	F: CATCGATATGGAGCTGGTGAAG
	R: GCCGTAATTTGGACAGGATCTG
TNF-α	F: CCTACCTGCTTCTCACCCATACC
	R: TTGATGGCAGAGAGAAGGTTGA
INF-γ	F: ATTTCGGTCAATGACGAGCAT
	R: GTTTCCTCTGGTTCGGTGACA

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Quantification of CMV copy number

DNA was isolated *via* the QIAamp® DNA Mini kit (Qiagen, Valencia, CA) according to manufacturer's protocol. DNA quality and concentration were determined using the BioMate 3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Absolute quantification of initial CMV copy number per 1 μg starting DNA was performed in triplicate using SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) and primers specific for the CVM promoter (Table IB) on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A standard curve ranging from 10^7 to 10^1 starting particles was prepared using the TV plasmid. If amplification was not detected, negative