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B. Gao[1,2#], W. Chen[2#*], L. Hao[1,2], G. Zhu[2], S. Feng[2], H. Ci[2], X. Zhou[1], P. Stashenko[3], and Y.P. Li[2*]

[1]The State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Sichuan, People's Republic of China; [2]Department of Pathology, University of Alabama at Birmingham, SHEL 810, 1825 University Blvd., Birmingham, AL 35294-2182, USA; and [3]Department of Immunology and Infectious Disease, The Forsyth Institute, Cambridge, MA, USA; [#]authors contributed equally as first authors; *corresponding authors, ypli@uab.edu; wechen@uab.edu

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Inhibiting Periapical Lesions through AAV-RNAi Silencing of Cathepsin K

ABSTRACT

Dental caries, one of the most prevalent infectious diseases worldwide, affects approximately 80% of children and the majority of adults. Dental caries may result in endodontic disease, leading to dental pulp necrosis, periapical inflammation and bone resorption, severe pain, and tooth loss. Periapical inflammation may also increase inflammation in other parts of the body. Although many studies have attempted to develop therapies for this disease, there is still an urgent need for effective treatments. In this study, we applied a novel gene therapeutic approach using recombinant adeno-associated virus (AAV)-mediated RNAi knockdown of Cathepsin K (Ctsk) gene expression, to target osteoclasts and periapical bone resorption in a mouse model. We found that AAV-sh-Cathepsin K (AAV-sh-Ctsk) impaired osteoclast function *in vivo* and furthermore reduced bacterial infection-stimulated bone resorption by 88%. Reduced periapical lesion size was accompanied by decreases in mononuclear leukocyte infiltration and inflammatory cytokine expression. Our study shows that AAV-RNAi silencing of Cathepsin K in periapical tissues can significantly reduce endodontic disease development, bone destruction, and inflammation in the periapical lesion. This is the first demonstration that AAV-mediated RNAi knockdown gene therapy may significantly reduce the severity of endodontic disease.

KEY WORDS: endodontic disease, inflammation, bone resorption, dental caries, osteoclast, gene therapy.

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INTRODUCTION

As infection in pulp tissue spreads throughout the root canal system toward the apical foramen and into the periodontal ligament (PDL), it inevitably leads to periapical bone resorption (Kakehashi *et al*., 1965; Nair, 1997; Stashenko, 1990). Endodontic therapy in the clinical setting removes necrotic infected pulp tissue and prevents this local inflammatory reaction. This treatment has a high degree of success (approximately 80%), but even when the highest standards are followed, endodontic failures still occur, and a complete bone healing or reduction of the apical lesion may not occur in all root-canal-treated teeth (Nair, 2004). It is known that osteoclasts function as the primary cell that mediates periapical bone resorption (Nair, 1997, 2004). It has also been established that receptor activator of nuclear factor-κβ ligand (RANKL), which stimulates osteoclast differentiation, is expressed by human dental pulp cells (Kojima *et al.*, 2006; Yamaguchi *et al*., 2006; Uchiyama *et al*., 2009). In addition, T- and B-cells may express RANKL (Kawai *et al.*, 2006; Lin *et al.*, 2011), although the contributions of these cell types to the stimulation of osteoclastogenesis and activation of osteoclasts in periapical bone resorption have yet to be determined.

Cathepsin K (Ctsk) is a lysosomal cysteine protease of the peptidase C1 protein gene family. It is strongly expressed by osteoclasts and plays an essential role in osteoclast function and the degradation of protein components of the bone matrix. Cathepsin K has also been shown to be involved in the regulation of Toll-like receptor 9 signaling in dendritic cells (Asagiri *et al.*, 2008). Mutations in Cathepsin K result in the human syndrome pycnodysostosis, in which bone resorption is impaired (Gelb *et al.*, 1996).

The aim of the current investigation was to determine conclusively the therapeutic potential of silencing Cathepsin K *in vivo* using adeno-associated virus (AAV)-sh-Cathepsin K (AAV-sh-Ctsk), to reduce endodontic disease progression, bone resorption, and inflammation in periapical lesions in a well-established mouse model. In addition, the current study aimed to evaluate AAV as a viable strategy for successful RNAi gene delivery. The AAV silencing approach is a relatively new and effective tool that has been proven successful in humans in a clinical setting (Carter, 2005). In addition, this approach is safe and well-tolerated by patients with advanced Parkinson's disease, suggesting that *in vivo* gene therapy is practical and causes only a very mild immune response to the AAV vector (Kaplitt *et al.*, 2007). Therefore, in this study we used the AAV RNAi knockdown system to investigate the therapeutic potential of Cathepsin K silencing, due to its unique attributes as described.

MATERIALS & METHODS

For complete Materials & Methods, please see the online Appendix.

Animals

Eighty-four six-week-old female WT BALB/cJ mice, purchased from the Jackson Laboratory (Bar Harbor, ME, USA), were used in this study. All experimental protocols were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (Animal Protocol Number 11090909236). This research conforms to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. Animals were sacrificed by CO_2 inhalation on day 42 after pulpal infection, and the mandibles were removed and prepared for analysis.

Cells and Cell Culture

Pre-osteoclasts and mature osteoclasts in primary culture were generated from mouse bone marrow (MBM) as previously described (Yang and Li, 2007; Feng *et al.*, 2009).

Design and Construction of shRNA

Using the Dharmacon siDESIGN centre (http://www.dharmacon.com) as described (Feng *et al*., 2009), we generated shRNA that would target Cathepsin K. As a control vector, we used the yellow fluorescent protein (YFP) expressing AAV-H1-shRNA-luc-YFP (gift from Dr. Sonoko Ogawa, University of Tsukuba, Japan), which contains a luciferase-specific shRNA and a YFP cassette (Musatov *et al*., 2006; Alexander *et al.*, 2010).

AAV-shRNA Viral Production and Purification

We used the AAV2 serotype and subcloned shRNA targeting Cathepsin K into the AAV-H1 vector (Musatov *et al*., 2006). The AAV Helper-Free System (AAV Helper-Free System Catalog #240071, Stratagene, La Jolla, CA, USA) was used for viral production with a triple-transfection, helper-free method, and was purified according to a modified published protocol (Hommel *et al.*, 2003).

Bacterial Infection and Transduction of AAV Vectors

Bacterial culture and infection procedure protocols were conducted as described (Sasaki *et al*., 2000). In brief, exposed dental pulps of mandibular first molars were infected with a mixture of 4 common human endodontic pathogens, including *Prevotella intermedia* [American Type Culture Collection (ATCC) 25611; Manassas, VA, USA], *Fusobacterium nucleatum* (ATCC 25586), *Peptostreptococcus micros* (ATCC 33270), and *Streptococcus intermedius* (ATCC 27335). The dental pulps of the mandibular first molars were exposed as described (Stashenko, 1990). Transduction consisted of injecting the viral vectors and PBS in a site-specific manner as described, with modifications (Musatov *et al*., 2006). Briefly, on day 1 and day 3 after pulpal infection, the mice were anesthetized *via* peritoneal injection of ketamine and xylazine. The right and left mandibular first molars were the sites of local injection of the vectors into the periapical tissues.

Data Quantification and Statistical Analyses

Experimental data are reported as mean ± SD of triplicate independent samples. The Figs. are representative of the data (n = 21 samples). Data were analyzed with the two-tailed Student's *t* test. The p values < 0.05 were considered significant.

RESULTS

Inhibitory Effect of AAV-sh-Cathepsin K Knockdown on Expression of Cathepsin K and Osteoclast-mediated Bone Resorption *in vitro*

To evaluate the effect of inhibition of Cathepsin K on bone resorption, we generated shRNA that targeted the expression of this enzyme. Cell fluorescence indicated that efficient transduction of pre-osteoclasts and osteoclasts with AAV-sh-Cathepsin K (AAV-sh-Ctsk) or AAV-sh-luc-YFP was achieved with a titer of approximately 6×10^{11} DNase-resistant particles (DRP)/mL (Fig. 1A). To confirm the effect of AAV-sh-Cathepsin K on gene silencing, we performed Western blot analysis, and found that osteoclasts transduced with AAV-sh-Cathepsin K had an approximately 80% reduction in Cathepsin K expression compared with osteoclasts transduced with AAV-sh-luc-YFP (control for shRNA and AAV) (Figs. 1B, 1C). These results indicate that AAV-sh-Cathepsin K targets Cathepsin K mRNA efficiently and reduces Cathepsin K protein expression.

To establish the effect of AAV-mediated Cathepsin K knockdown on osteoclast function *in vitro*, we analyzed bone resorption using wheat germ agglutinin (WGA) and scanning electron microscopy (SEM) to visualize resorption pits on bone (Fig. 1D, middle and bottom panels). As shown, AAV-mediated knockdown of Cathepsin K was highly effective in impairing bone resorption compared with the control group ($p < 0.005$; Fig. 1E). We also performed acridine orange staining, which indicates extracellular matrix acidification. As expected, we found that osteoclasts transduced with AAV-sh-Cathepsin K showed no difference in extracellular acidification compared with osteoclasts transduced with no vector (mock group) or with AAV-sh-luc-YFP (Fig. 1D, top panel), demonstrating the specificity of the Cathepsin K inhibition.

AAV-sh-Cathepsin K Effectively Transduces Periapical Tissue *in vivo* and Protects Mice from Bone Loss Due to Endodontic Infection

Fluorescent analysis of the infected mice treated with AAV-sh-Cathepsin K compared with that of the uninfected control (Normal) group demonstrated that the AAV vectors successfully infiltrated the periapical tissues (Fig. 2A). To determine the efficacy of AAV-sh-Cathepsin K in reducing the extent of endodontic disease, we used a model of periapical lesion induction as described (Sasaki *et al*., 2000). Exposed mandibular first molar dental pulps were infected with a mixture of 4 common endodontic pathogens: *Prevotella intermedia, Fusobacterium*

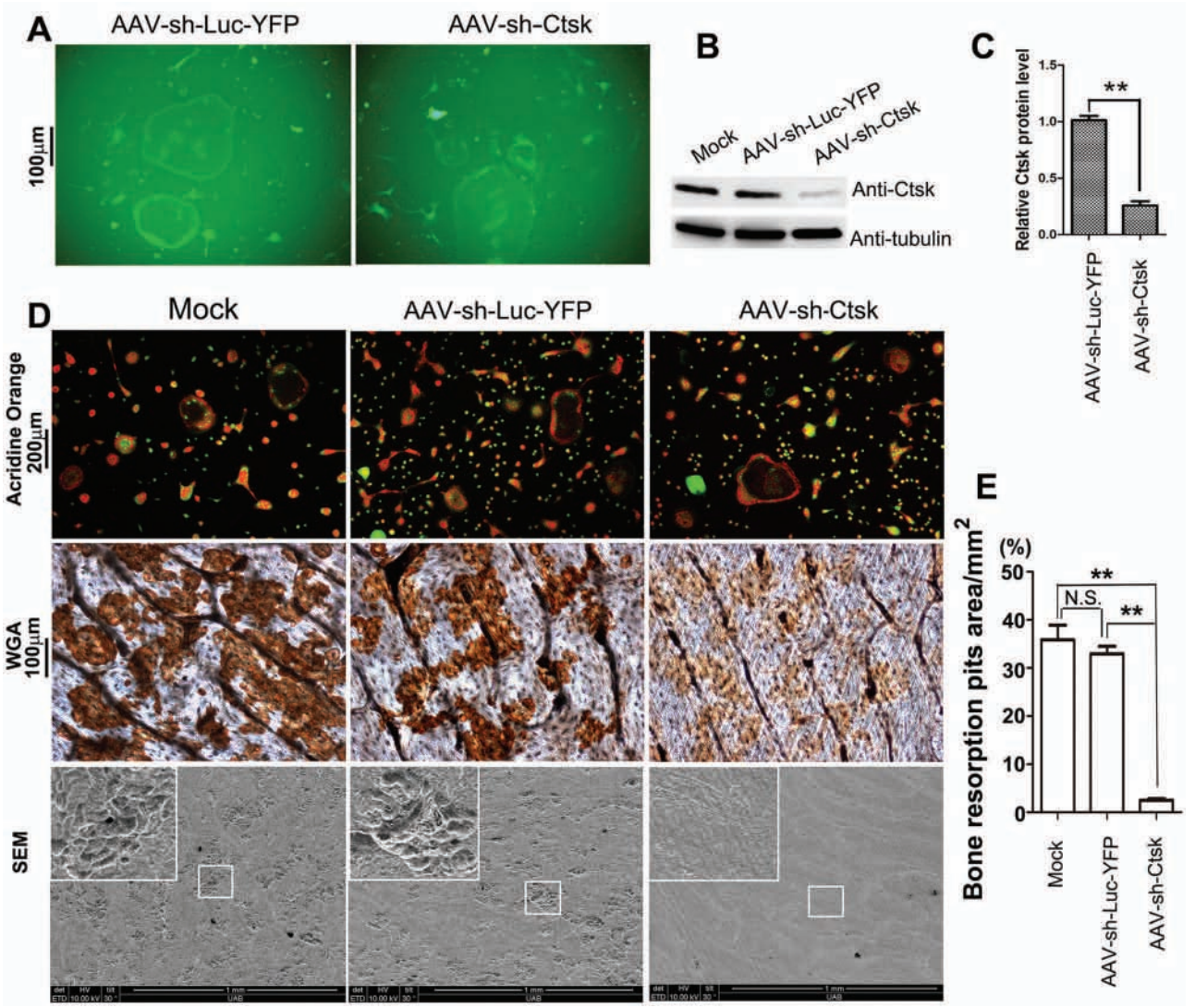

Figure 1. AAV-sh-Cathepsin K efficiently knocked down expression of Cathepsin K and impaired osteoclast-mediated bone resorption *in vitro*. **(A)** Immunofluorescent photomicrograph of AAV-sh-luc-YFP and AAV-sh-Cathepsin K treatment groups 7 days after transduction. The experiments were performed in triplicate. **(B)** Western blot of Cathepsin K expression in murine bone marrow cells stimulated with M-CSF/RANKL for 3 days to induce differentiation of osteoclasts, that were then transduced with no vector (mock), AAV-sh-luc-YFP (control vector), or AAV-sh-Cathepsin K. **(C)** Quantification of Western blot analysis demonstrates that the AAV-sh-Cathepsin K treatment group significantly reduced expression of Cathepsin K as compared with the AAV-sh-luc-YFP treatment group. **(D)** Untransduced osteoclasts (Mock) and osteoclasts transduced with AAV-sh-luc-YFP (control) or AAV-sh-luc-Cathepsin K stained with acridine orange to show extracellular acidification (top panel). Resorption pits on bone slices were visualized by WGA (middle panel) and scanning electron microscopy (SEM) (bottom panel). The experiment was performed in duplicate on 4 independent occasions. **(E)** Quantification of resorption pits on bone slices was significantly lower in the AAV-sh-Cathepsin K treatment group compared with the Mock and AAV-sh-luc-YFP groups. All assays were performed in triplicate; a representative photomicrograph from each assay is shown. N.S., Not Significant. $**p < 0.01$.

nucleatum, Peptostreptococcus micros, and *Streptococcus intermedius*. Radiographic imaging revealed that the infected group treated with the control AAV-sh-luc-YFP had significant periapical bone resorption surrounding the distal root of the mandibular first molar compared with the uninfected control (Normal) group. In contrast, the AAV-sh-Cathepsin K treatment group showed minimal bone resorption, which was comparable with that in the Normal group (Fig. 2B). This was confirmed by detailed microCT analysis of Bone Volume (BV)/Total Volume (TV) ratios of the periapical area surrounding the distal root of the first molars, which showed that infected mice treated with AAV-sh-Cathepsin K had an 88% reduction in infection-stimulated bone resorption (Fig. 2D), compared with infected mice treated with the AAV-sh-luc-YFP control vector (Fig. 2C).

AAV-sh-Cathepsin K Decreases Mononuclear Leukocytes and T-cells in Periapical Tissues

Hematoxylin and eosin (H&E) staining indicated that periapical tissue sections from infected mice treated with AAV-sh-Cathepsin K had significantly fewer mononuclear leukocytes, as indicated by nuclear morphology, compared with infected mice

treated with AAV-sh-luc-YFP (34 ± 10 *vs.* 112 ± 8 *per* section, respectively; $p < 0.001$) or uninfected controls (17 ± 2) (Fig. 3A). Immunofluorescence staining revealed that the number of CD3+-expressing T-cells in periapical tissues showed a parallel reduction in the AAV-sh-Cathepsin K treatment group compared with the AAV-sh-luc-YFP group (39 ± 9 *vs*. 93 ± 8; $p < 0.001$) or uninfected controls (27 ± 2), indicating that AAV-sh-Cathepsin K also reduced T-cells *in vivo* (Fig. 3B). These results indicate that, in addition to inhibiting bone resorption, AAV-sh-Cathepsin K had the collateral effect of reducing inflammatory cell infiltration.

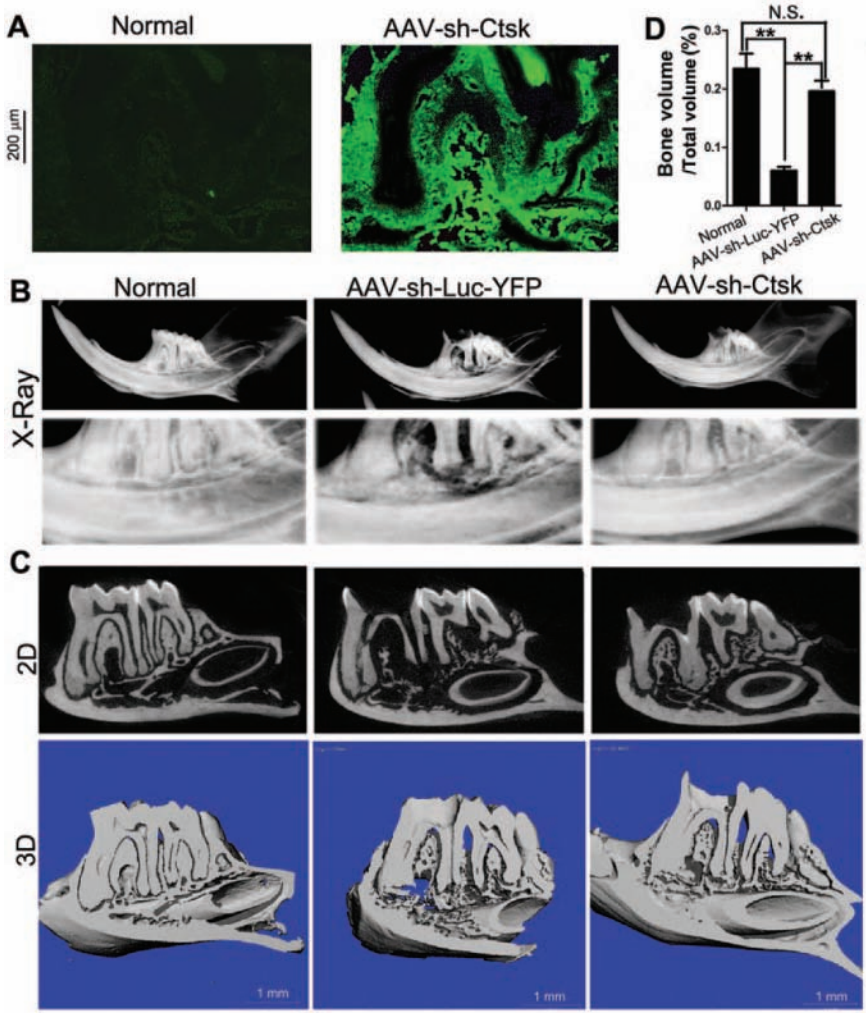

Figure 2. AAV effectively transduced endodontic tissue *in vivo* and protected mice from bone loss due to endodontic infection. (**A**) Fluorescent micrographs of eGFP expression by AAV-infected cells in uninfected (Normal) mice and infected mice treated with AAV-sh-Cathepsin K. The experiments were performed in triplicate. (**B**) Radiographic imaging of the crown and distal root of the mandibular first molar in uninfected mice (Normal), infected mice treated with AAV-sh-luc-YFP, and infected mice treated with AAV-sh-luc-Cathepsin K. Severe periapical bone loss is visible in the AAV-sh-luc-YFP image compared with the minimal bone loss in the AAV-sh-Cathepsin K image (n = 21). (**C**) µCT analysis of the mandibular first molar in uninfected mice (Normal), infected mice treated with AAV-sh-luc-YFP, and infected mice treated with AAV-sh-Cathepsin K. Severe periapical bone loss is visible in the AAV-sh-luc-YFP image compared with the minimal bone loss in the AAV-sh-Cathepsin K image (n = 21). (**D**) Quantification of Bone Volume (BV)/Total Volume (TV) from uninfected (Normal) mice, infected mice treated with AAV-sh-luc-YFP, and infected mice treated with AAV-sh-Cathepsin K. N.S., Not Significant. $^{**}p < 0.01$ (n = 21).

AAV-sh-Cathepsin K Reduces Expression of Inflammatory Mediators in Periapical Tissues

Investigation of the inflammation status of periapical tissues by qRT-PCR analysis revealed that the AAV-sh-Cathepsin K treatment group had significantly lower mRNA expression of the inflammatory mediators Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), and Interleukin-17α (IL-17α) compared with the AAV-sh-luc-YFP group ($p < 0.05$) (Fig. 4A). The expression of tartrate-resistant acid phosphatase 5 (Acp5), which is an established marker of osteoclasts *in vivo*, and the macrophage marker CD115 (M-CSF receptor) was also significantly lower ($p < 0.01$ for Acp5; $p < 0.05$ for CD115) in the AAV-sh-Cathepsin K treatment group, albeit they were not as dramatically reduced, indicating that osteoclasts were present in lesions in all infected mice (Fig. 4A). To confirm the effect of Cathepsin K silencing on inflammatory cytokines at the protein level, we performed enzyme-linked immunosorbent assays (ELISA). The results showed that both infected groups had elevated levels of IL-1α, Interleukin-12α (IL-12α), IL-17α, and tumor necrosis factor-α (TNF-α), compared with uninfected controls. However, in all cases, the levels of these mediators were lower in the AAV-sh-Cathepsin K treatment group compared with the AAV-sh-luc-YFP group (Fig. 4B).

DISCUSSION

Endodontic therapy in the clinical setting is directed toward the removal of necrotic, infected pulp tissue, to inhibit the local inflammatory reaction in periapical tissues, thereby permitting the formation of reparative bone. Bone healing, even in cases of successful treatment, takes 6 mos or longer; hence an earlier reversal of the resorptive process would be important for the resolution of the periapical lesion. In this study, we tested a novel gene therapy approach to this problem using AAV2 as a vector to knock down the osteoclast-expressed enzyme Cathepsin K, to reduce bone resorption induced by pulpal infection. Our results demonstrated that the AAV vector efficiently

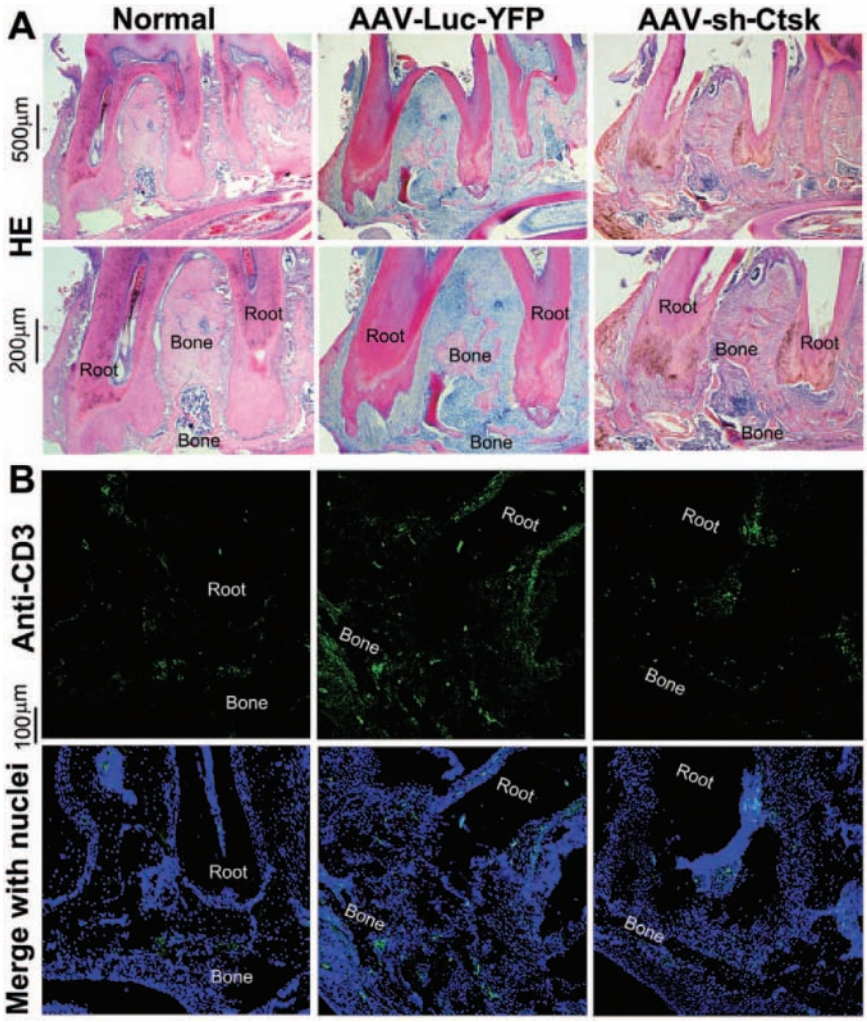

Figure 3. AAV-sh-Cathepsin K significantly decreased cell infiltration and bone resorption in periapical tissues. (**A**) Hematoxylin and eosin (H&E) staining of sections from untreated, uninfected mice (Normal), infected mice treated with AAV-sh-luc-YFP (Control), and infected mice treated with AAV-sh-Cathepsin K. Bone resorption and mononuclear cell infiltration was significantly decreased in the AAV-sh-Cathepsin K treatment group compared with the control AAV-sh-luc-YFP group (n = 21). (**B**) Immunofluorescence staining of periapical lesion sections indicates that uninfected mice (Normal) and infected mice treated with AAV-sh-Cathepsin K have fewer CD3-positive (green) T-cells compared with infected mice treated with AAV-sh-luc-YFP. Cell nuclei were labeled with DAPI DNA stain (blue). The experiments were performed in triplicate.

suppressed Cathepsin K and reduced osteoclast bone resorption *in vitro*, but did not affect extracellular acidification by these cells. Remarkably, transduction of periapical lesions with AAV-sh-Cathepsin K profoundly reduced infection-stimulated bone resorption by 88% *in vivo*. The reduction in bone resorption and lesion size resulted in fewer infiltrating mononuclear cells, including T-cells, and lowered expression of inflammatory mediators. This study is the first to report the successful use of AAV-mediated gene knockdown as a gene therapy strategy for periapical inflammatory disease. Since patients with endodontic disease suffer from both inflammation-induced tissue damage and bone loss, gene therapy against a single target that can dramatically inhibit both disease manifestations may have potential as a therapeutic approach in humans.

Gene therapy with AAV vectors has previously been reported in models of periodontal disease, which, like endodontic disease, have a microbial etiology. Systemic transduction with TIMP-4 was reported to reduce both adjuvant-induced arthritis and periodontitis in rats (Ramamurthy *et al*., 2005). Inhibition of periodontal bone loss was also reported following AAV gene transfer of mitogen-activated protein kinase phosphatase-1 (MKP-1), which dephosphorylates MAPKs and inhibits immune responses (Yu *et al*., 2011). Transduction of gingival tissues with AAV/2/1-TNF Receptor: Fc reduced *P. gingivalis*-induced periodontal bone loss by approximately 50% in a mouse model, with parallel reductions in pro-inflammatory cytokines and osteoclasts (Cirelli *et al*., 2009). In the latter study, the AAV-TNFR:Fc vector was delivered 3 times *per* wk for 8 wks, compared with 2 injections of AAV-sh-Cathepsin K in our protocol. The greater efficiency of cathepsin K inhibition (88%) may reflect the critical importance of this enzyme for osteoclast function, given that humans and mice with cathepsin K deficiency are osteopetrotic ('pycnodysostosis'), with highly compromised resorption due to an inability to degrade the collagen matrix of bone (Gelb *et al.*, 1996; Chen *et al*., 2007). Thus, the direct targeting of osteoclasts may be the most effective strategy for gene therapy aimed at inhibiting bone loss. Another factor in the efficiency in the endodontic *vs*. periodontal models may be the better localization of the AAV vector to the confines of the periapical lesion *vs*. the gingiva, which furthermore requires delivery at multiple sites. No systemic effects were observed in mice following treatment with AAV-sh-Cathepsin K. Furthermore, osteoclasts transduced with AAV-sh-Cathepsin K showed no difference in extracellular acidification compared with osteoclasts transduced with no vector (mock group) or with AAV-sh-luc-YFP, demonstrating the specificity of the Cathepsin K inhibition.

In a non-oral infection model, AAV-mediated gene transfer of IL-10 reduced airway inflammation in mice infected with *Pseudomonas aeruginosa*, the major pathogen in cystic fibrosis (Buff *et al*., 2010). In bone resorption models, osteoprotegerin (OPG) gene transfer attenuated osteoclast number and activity in debris-induced osteolysis (Ulrich-Vinther, 2007) and reduced aseptic joint prosthesis loosening (Zhang *et al*., 2010). Collagen-induced arthritis and inflammatory responses were attenuated by AAV-IL-4 (Cottard *et al.*, 2000). In general,

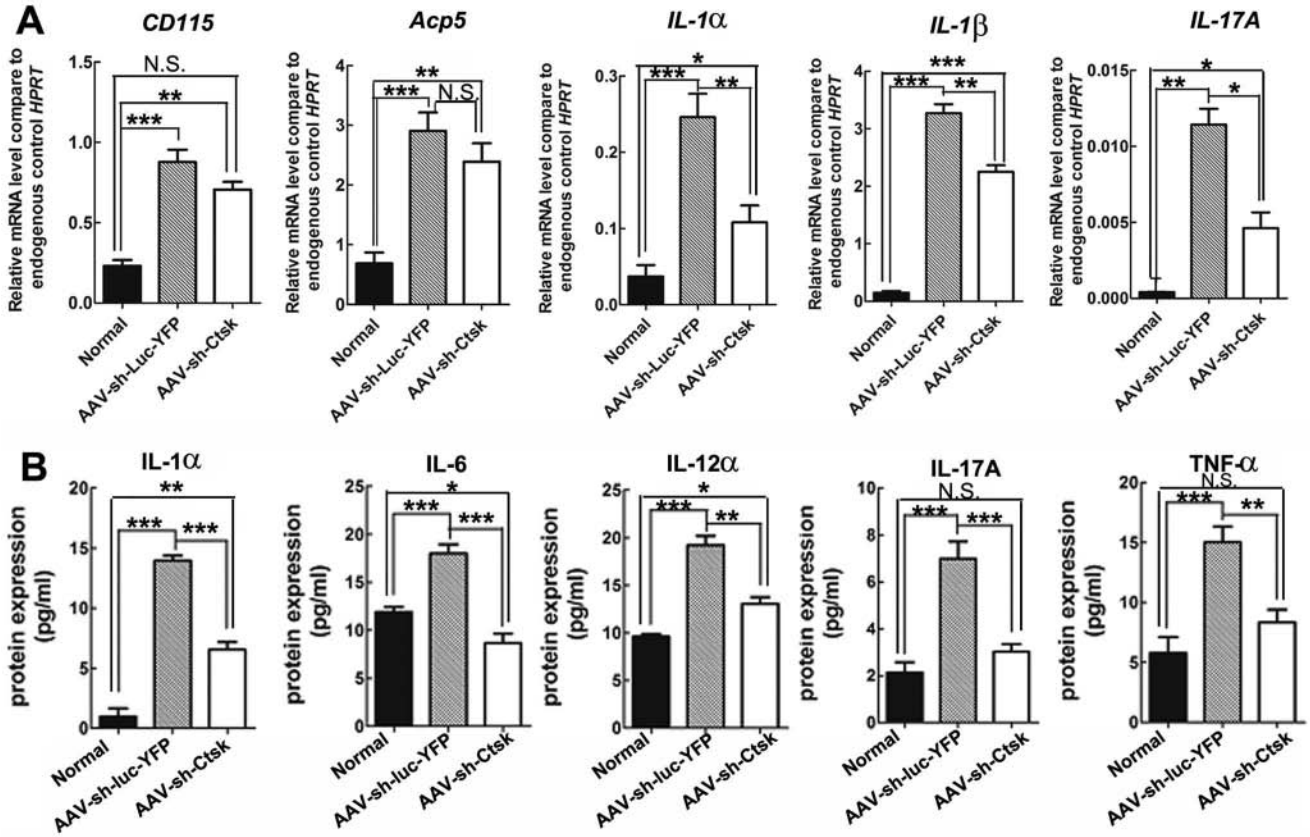

Figure 4. AAV-sh-Cathepsin K reduced the expression of inflammatory mediators in periapical tissues. (**A**) qRT-PCR results for osteoclast differentiation marker (CD115 Acp5) and inflammatory mediator (IL-1α, IL-1β, IL-17α) gene expression, in the periapical tissues of normal uninfected mice and infected mice treated with AAV-sh-luc-YFP or with AAV-sh-Cathepsin K. Expression levels were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (Hprt) (n = 21). (**B**) Protein levels of IL-1α, IL-6, IL-12α, IL-17α, and TNF-α in periapical lesions as detected by ELISA. N.S., Not Significant. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$ (n = 21).

the direct targeting of osteoclasts by AAV-sh-Cathepsin K appears to be both more specific and more efficient in reducing inflammatory bone resorption, compared with indirect approaches that target inflammatory mediators and indirectly regulate osteoclasts.

Osteoclast markers including Acp5 were reduced by AAV-sh-Cathepsin K, as was CD115, a marker for macrophages and osteoclast precursors. Similarly, all cytokines examined showed reduced levels in periapical tissues in AAV-sh-Cathepsin K-treated animals. These mediators (IL-1α, IL-1β, IL-17α) have all been previously identified in periapical lesion tissues (Stashenko, 1990; Wang and Stashenko, 1993; Kamolmatyakul *et al.*, 2004; Oseko *et al.*, 2009). Since the block sections taken for analysis included bone, tooth roots, and periapical tissues, it is likely that the reduced lesion size in the AAV-sh-Cathepsin K group accounts for the observed reductions in both osteoclasts and cytokines. Alternatively, recent evidence suggests that cathepsin K may regulate immune and inflammatory responses, in part *via* Toll-like receptor 9 signaling (Asagiri *et al.*, 2008). Additional studies are needed to distinguish between these possibilities.

In summary, our findings demonstrate that inhibition of osteoclast function *via* AAV-mediated gene therapy may be a viable therapeutic approach to the resolution of inflammatory bone loss in endodontic diseases.

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