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Inhibiting Periapical Lesions through AAV-RNAi Silencing of Cathepsin K

APPENDIX

MATERIALS & METHODS

Animals

The animals were housed in the University of Alabama at Birmingham (UAB) animal facility and were given food and water *ad libitum*. All experimental protocols were completed within 16 wks after the animals were born (Tomar *et al.,* 2003; Kumamoto *et al*., 2006).

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). Briefly, MBM was obtained from tibiae and femora from six-week-old female WT BALB/cJ mice. MBM cells (1-2 × 10^5) were seeded into wells of a 24-well plate, and 1 × 10^6 MBM cells were seeded into wells of a 6-well plate. MBM cells were cultured in α-modified Eagle's medium (α-MEM; GIBCO-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; GIBCO-BRL) containing 10 ng/mL M-CSF (R&D Systems, Minneapolis, MN, USA). After 24 hrs, cells were cultured in the presence of 10 ng/mL RANKL (R&D Systems) and 10 ng/mL M-CSF for an additional 96 hrs (totaling 120 hrs of RANKL/M-CSF stimulation) to generate mature osteoclasts.

Design and Construction of shRNA

AAV-H1 (gift from Dr. Sonoko Ogawa) contains a human Pol III H1 promoter for the expression of shRNA as well as an independent green fluorescent protein (eGFP) expression cassette. A BLAST homology search predicted that these shRNAs would not affect Cathepsin K or any other known mouse gene. We cloned the H1 promoter shRNA expression cassette into the AAV construct as described (Hommel *et al*., 2003; Tomar *et al.,* 2003; Musatov *et al.,* 2006). The following oligonucleotide was annealed and cloned downstream of the H1 promoter of AAV-H1 into BglII and HindIII sites to produce AAV-H1-shRNA Cathepsin K: (5′-GATCCCCGAGGTGTGTACTATGATGAAA**TTCAAGAGA**TTTCATCATAGTACACACCTCTTTTTGGAAT-3′). Nucleotides specific for targeting Cathepsin K, located within exon 7 on Cathepsin K and all its isoforms, are underlined. The bold type signifies the 9-bp hairpin spacer.

AAV-shRNA Viral Production and Purification

We used the AAV Helper-Free System (AAV Helper-Free System Catalog #240071, Stratagene) for viral production using a triple-transfection, helper-free method, and purified the virus using a modified published protocol (Hommel *et al*., 2003). Briefly, HEK 293 cells were cultured in 10 150 × 25-mm cell culture dishes and transduced with pAAV-shRNA, pHelper, and pAAV-RC plasmids (Stratagene) by a standard calcium phosphate method. Cells were collected after 60 to 72 hrs and subjected to lysis *via* shaking with chloroform at 37°C for 1 hr. Sodium chloride was then added, and the cells were shaken at room temperature for 30 min. The stock was then spun at 12,000 RPM for 15 min, and the supernatant was collected and cooled on ice for 1 hr with PEG8000. The solution was spun at 11,000 RPM for 15 min, and then the pellet was treated with DNase and RNase. After the addition of chloroform and a 5-minute centrifugation at 12,000 RPM, the purified virus was in the aqueous phase at viral particle numbers of 1 x 10^{10}/mL. The AAV particle titer was determined by an AAV Quantitation Titer Kit (Cell Biolabs, Inc., San Diego, CA, USA). The viral vectors were evaluated for transduction efficiency as previously described (Tu *et al.,* 2009). Briefly, optimal viral particle numbers for infection were determined from the percentage of target cells expressing YFP. To confirm the effect of silencing, we examined the expression of Cathepsin K in osteoclasts using Western blot and immunofluorescence techniques.

Bacterial Culture and Infection

Bacterial culture and infection procedure protocols were conducted as described (Sasaki *et al*., 2000). All 4 species of

bacteria (*Prevotella intermedia*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, and *Streptococcus intermedius)* were cultured under strict anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) in broth over 7 to 8 days. Microbes were harvested, re-suspended, and evaluated for cell concentration of each species *via* optical density readings. The 4 organisms were then mixed for a total of 10^{10} cells/species/mL PBS in 3% methylcellulose. At the time of pulp exposure, animals were infected with 10 μL of the polymicrobial solution placed inside the access opening of each molar and carried to the periapical tissues by means of a #8 endodontic file.

Pulp Exposure and Transduction of AAV Vectors

Pulp exposure was performed as described (Stashenko, 1990; Kawashima *et al.,* 1999). In brief, mice were anesthetized by the intraperitoneal injection of 62.5 mg/kg ketamine and 12.5 mg/kg xylazine. The dental pulps of the mandibular first molars were exposed with a ¼ round carbide bur powered by a variable speed electric rotary handpiece (Osada Electric, Los Angeles, CA, USA) under a surgical microscope (model MC-M92; Seiler, St. Louis, MO, USA). After the roof of the pulp chamber was removed, the size of the exposure was approximately 1.5 to 2.0 mm in diameter. We used stainless steel hand files #8 (*Dentsply*/Maillefer, Johnson City, TN, USA) and stainless steel rotary file #15 (*Dentsply*/Maillefer) to establish canal patency. After exposed pulps were infected with a mixture of 4 common human endodontic pathogens, access openings were left open to the oral environment for 24 hrs. Transduction consisted of injecting the viral vectors and PBS in a site-specific manner as described, with some modification (Musatov *et al.,* 2006). Briefly, on day 1 and day 3 after the pulpal infection, the mice were anesthetized *via* peritoneal injection as described above. The right and left mandibular first molars were the sites of local injection of the vectors into the periapical tissues. Using a 5-μL syringe with a 31-gauge needle, we injected approximately 3 μL (2×10^9 packaged genomic particles in PBS) of either AAV-sh-Cathepsin K (n = 21) or AAV-sh-luc-YFP (n = 21) through the apical foramen and into the apical periodontium. As a negative control (Normal), mice (n = 21) were not exposed to or treated with either of the viral vectors. As a positive control (Disease), mice (n = 21) were exposed to and infected, but not treated with, either viral vector. All exposure sites were then filled with self-cure composite temporary filling after AAV transduction until the end of the observation period.

Harvest and Preparation of Tissue Samples

Animals were sacrificed by CO_2 inhalation on day 42 after infection. The mandibles were removed and hemisected. The samples from the left side were de-fleshed in 2.6% sodium hypochlorite for bone measurement analysis. The concentration of sodium hypochlorite was determined according to a pilot study conducted for the purpose of developing a protocol for removing the soft tissue from the bone, as previously outlined (Trepagnier *et al*., 1977). The left tissue blocks were soaked in 2.6% NaOCl for 30 to 40 min, rinsed in tap water 3 times, placed in 70% alcohol, stained with 1% methylene blue, and then mounted on microscope slides for bone loss measurements. The jaw samples from the right side were immediately fixed in 4% paraformaldehyde prepared for histological analysis according to a standard protocol with modification for samples that were prepared for cryostat-frozen sectioning. In brief, samples specified for frozen sections were fixed in 4% formaldehyde for 24 hrs, washed with PBS, decalcified in 10% EDTA for 10 days (EDTA replenished each day), soaked in 30% sucrose for 24 hrs, submerged in frozen section compound (FSC 22, Surgipath, Leica Microsystems, Wetzlar, Germany), stored at -20ºC, and then stored at -80ºC prior to cryostat sectioning.

Micro-computed Tomography Analysis

Micro-computed tomography (μCT) scans were evaluated for bone loss as described (Balto *et al.,* 2000). In brief, the most centrally located radiographic section that provided a view of the crown and distal root of the mandibular first molar and that exhibited a patent root canal apex was selected for quantification of periapical bone loss. The cross-sectional area of the periapical lesions was selected *via* Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Bone loss was also evaluated through three-dimensional μCT analyses as described (Balto *et al.,* 2002; von Stechow *et al.,* 2003). Briefly, the section that showed the largest root canal space was selected as the 'pivot section' for quantification of periapical bone loss.

A detailed description of the selection of the region of interest for volumetric analysis follows:

(1) Sample preparation: The mandibular samples were first fixed in 4% paraformaldehyde (PFA) overnight, then kept in 70% ethanol before application. The fixed samples were then analyzed by means of compact fan-beam-type tomography (μCT 40; Scanco Medical, Bassersdorf, Switzerland). The mandibles were placed in an open cylindrical sample holder. Approximately 101 microtomographic slices with an increment of 12 μm were acquired, covering the entire buccal-lingual thickness of the sample. Void space was segmented from bone and dentin by a global thresholding procedure.

(2) Image analysis: For the 3-D analysis of data, a volumetric model was used, and the lesion and control void spaces were defined by means of a contouring tool to provide accuracy. The sample was placed in a buccal-lingual direction. The analysis began with the first longitudinal section showing the root and was completed with the last section in which the root was completely surrounded by lingual alveolar bone. The same area of periapical destruction surrounding the distal root was selected. In the selected contoured area, basic structures were measured by bone volume (BV), total volume (TV), and BV to TV ratio (BV/TV).

(3) Statistical analysis: The value obtained by μCT was evaluated among the normal, AAV-sh-luc-YFP, and AAV-sh-Atp6i groups by one-way ANOVA tests. The data were analyzed with Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

(4) Parameter: segmentation 0.7/1/210.

Histological Analysis

Tissue samples were prepared for either frozen or paraffin sections. For paraffin section, tissues were fixed in 4% paraformaldehyde for

Appendix Table. qRT-PCR Primer Numbers

Gene Symbol	Applied Biosystems Assay ID
Csf1r (CD115)	Mm01266652_m1
Acp5	Mm00475698_m1
Il1a (IL-1α)	Mm00439620_m1
Il1b (IL-1β)	Mm01336189_m1
Il17a (IL-17α)	Mm00439618_m1

24 hrs, and decalcified by 10% EDTA for 10 days, with solution replaced each day; dehydrated with 50, 70, 95, and 100% alcohol, for 2 hrs each; washed in paraffin acetone for 30 min in a 60ºC incubator; washed in chloroform for 30 min at 60ºC; and embedded in paraffin. After hematoxylin and eosin (H&E) staining, mononuclear leukocytes in lesions were counted under high power by a blinded investigator. For the detection of bone-resorbing osteoclasts, TRAP staining was performed. Tissue sections were deparaffinized and hydrated through xylene and a graded alcohol series, pre-incubated with 50 mM sodium acetate and 40 mM potassium sodium tartrate buffer for 20 min, and incubated with TRAP substrate solution for glycerol jelly.

Western Blotting Analysis

Western blotting, performed as previously outlined (Yang *et al.,* 2003; Yang and Li, 2007), was visualized and quantified with a Fluor-S Multi-Imager equipped with Multi-Analyst software (Bio-Rad, Hercules, CA, USA). A rabbit anti-Cathepsin K antibody previously generated in our laboratory (Feng *et al.,* 2009) was used at a 1:1,000 dilution, with goat anti-rabbit IgG-HRP (7074S, Cell Signaling, Cambridge, MA, USA) used at a 1:5,000 dilution to visualize the reaction.

Immunofluorescence Analysis

We performed immunofluorescence analysis as outlined previously (Chen *et al*., 2007), with the exception that we used anti-Cathepsin K (Chen *et al*., 2007) rabbit polyclonal anti-CD3 (Abcam, Cambridge, MA, USA) as the primary antibodies and rhodamine red-X-labeled secondary antibodies (1:1,000 dilution). Data were documented by epifluorescence on a Zeiss Axioplan microscope in the Developmental Neurobiology Imaging and Tissue Processing Core at the UAB Intellectual and Disabilities Research Center. Nuclei were visualized with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole; Sigma, St. Louis, MO, USA). CD3 cell counts were done by a blinded investigator. The experiments were performed in triplicate.

Acridine Orange Staining

Osteoclast acid production was determined with acridine orange as described previously (Li *et al.,* 1999). Osteoclasts that had been transduced with viral vectors after 48 hrs of RANKL/M-CSF stimulation were incubated in α-MEM containing 5 μg/mL of acridine orange (Sigma) for 15 min at 37°C, washed, and rinsed for 10 min in fresh media without acridine orange. The cells were observed under a fluorescence microscope at 490-nm excitation filter and with a 525-nm arrest filter. The experiment was performed in duplicate on 4 independent occasions in a 24-well plate.

In vitro Bone Resorption Assays

Bone resorption activity was assessed as described (Sasaki *et al*., 2004) with minor modifications. MBM cells were cultured on bovine cortical bone slices in 24-well plates and transduced with viral vectors after a 48-hour induction by RANKL and M-CSF. The bone slices were harvested after 6 days, and the culture media were collected. Cells adhering to the bone slices were removed with 0.25 M ammonium hydroxide and mechanical agitation. Bone slices were subjected to scanning electron microscopy (SEM) with a Philips 515 SEM (Department of Materials Science and Engineering, UAB). We also assessed *in vitro* bone resorption using wheat germ agglutinin (WGA) to stain exposed bone matrix proteins as described (Muzylak *et al.,* 2007). The assays were performed in triplicate. The data were quantified by measurement of the percentage of the bone surface area resorbed in 3 random resorption sites, as determined with the ImageJ analysis software.

Real-time Quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed as described (Nijenhuis *et al*., 2008; Allaire *et al.,* 2011) with the use of TaqMan probes purchased from Applied Biosystems (Appendix Table) according to the manufacturer's instructions. Briefly, cDNA fragments were amplified by TaqMan® Fast Advanced Master Mix (Applied Biosystems). Fluorescence from each TaqMan probe was detected with the Step-One real-time PCR system (Applied Biosystems). The mRNA expression level of the housekeeping gene *hypoxanthine-guanine phosphoribosyl transferase* (*Hprt*) was used as an endogenous control and facilitated the calculation of specific mRNA expression levels as a ratio of *Hprt (deltaCT)*. qPCR reactions were performed under standard conditions and were repeated at least 3 times.

Enzyme-linked Immunosorbent Assay (ELISA)

To determine the effect of Cathepsin K knockdown on the levels of inflammatory cytokines in periapical tissues, we used ELISA as previously described (Sasaki *et al.,* 2000, 2004). Briefly, assays for cytokines in the tissue extracts were performed with commercially available ELISA kits obtained from the following sources: IL-1α (BioLegend, San Diego, CA, USA), IL-6 (eBioscience, San Diego, CA, USA), and IL-17α (eBioscience, San Diego, CA, USA). All assays were conducted in accordance with the manufacturer's instructions, with results expressed as pg cytokine/mg tissue.

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). Data were analyzed with the two-tailed Student's *t* test.

The p values < 0.05 were considered significant. Data quantification analyses were performed with the NIH ImageJ Program as described (Chen *et al*., 2007; Yang and Li, 2007). To calculate the percentage of protection, we used the difference between the BV/TV ratios of the Normal group and the AAV-sh-luc-YFP treatment group to represent 100% bacterial infection-stimulated bone resorption. We then calculated the difference between BV/TV ratios of the Normal group and AAV-sh-Cathepsin K treatment group. We calculated the difference between these 2 percentages to determine the percentage of protection from bacterial infection-stimulated bone erosion by AAV-sh-Cathepsin K treatment.

APPENDIX REFERENCES

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