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Mechanical force-mediated pathological cartilage thinning is regulated by necroptosis and apoptosis

C. Zhang †, S. Lin †, T. Li ‡, Y. Jiang †, Z. Huang †, J. Wen †, W. Cheng †, H. Li † *

† *Department of Orthodontics, Nanjing Stomatological Hospital, Medical School of Nanjing University, Nanjing, China*
‡ *Department of Biostatistics, Robert Stempel College of Public Health and Social Work, Florida International University, USA*

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SUMMARY

Objective: This study aimed to identify the mechanisms underlying mandibular chondrocyte cell death and cartilage thinning in response to mechanical force.
Material and methods: An *in vivo* model (compressive mechanical force) and an *in vitro* model (TNF-α+cycloheximide) were used to induce mandibular chondrocyte necroptosis. Hematoxylin and eosin staining and transmission electron microscopy were used to assess histological and subcellular changes in mandibular chondrocyte. Immunohistochemistry, western blotting, and real-time PCR were performed to evaluate changes in necroptotic protein markers. Cell activity, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) were examined *in vitro*.
Results: The expression of RIP1, RIP3 and Caspase-8 in mandibular chondrocytes significantly increased after 4 days of compressive mechanical force. Furthermore, the inhibition of necroptosis by Necrostatin-1 (Nec-1) or the inhibition of apoptosis by N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) partially restored mechanical force-mediated mandibular cartilage thinning and chondrocyte death. Moreover, a synergistic effect on cell death inhibition and mandibular cartilage thickness restoration were found when treated with Nec-1+Z-VAD. The results of the *in vitro* model were in line with the *in vivo* ones, indicating that the changes in MMP and ROS generation contributed to mandibular chondrocyte apoptosis and necroptosis.
Conclusion: In addition to apoptosis, necroptosis also plays critical roles in pathological changes in mandibular cartilage after compressive mechanical force stimulation, implying RIP1, a master protein that mediates both necroptosis and apoptosis, as a potential therapeutic target in temporal mandibular osteoarthritis.
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Introduction

Temporal mandibular joint osteoarthritis (TMJOA) leads to serious joint pain and disorders, which cause substantial damages to a patient's quality of life[1]. Previous studies have indicated that compressive overload mechanical stimulation triggers pathological changes in cartilage, including mandibular chondrocyte death, matrix degradation and cartilage thinning, while the underlying mechanisms are still poorly understood[2,3]. By using an *in vivo* mechanical force-load model of mandibular cartilage thinning, we previously observed that mandibular chondrocytes death was mediated partly by apoptosis[4,5], suggesting that other type(s) of cell death was (were) involved in the process.

In addition to apoptosis, necroptosis is another type of programmed cell death induced by death receptors, mitochondrial dysfunction or oxidative stress and which shares similar morphological characteristics to those of necrosis[6]. Necroptosis is activated when apoptosis is blocked by Z-VAD under certain circumstances; however, necroptosis may be predominant even when apoptosis is competent, indicating that is another essential and alternative/complementary mechanism of cell death[7]. Necroptosis has been shown to play critical roles in the pathogenesis of many diseases, including inflammation[8], ischemic injury[9] and neurodegeneration[10]. Receptor-interacting protein (RIP) kinases, especially RIP1 and RIP3, play important roles in the initiation and

* Address correspondence and reprint requests to: H. Li, Department of Orthodontics, Nanjing Stomatological Hospital, Medical School of Nanjing University, Nanjing, China. Fax: 86-025-83620173.
E-mail address: lihuang76@nju.edu.cn (H. Li).

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execution of necroptosis. Once activated by stimuli such as tumor necrosis factor α (TNF-α), RIP1 recruits RIP3 to form necrosome[11], which in turn promotes RIP3 targeting of MLKL to permeabilize plasma membranes and elicit inflammation[12,13].

The roles of TNF-α in chondrocyte death in OA have been well established[14–16]. Previous studies have primarily indicated that both apoptosis and necroptosis are induced in chondrocyte when treated with TNF-a and cycloheximide (CHX) or D469del-COMP retention *in vitro*[17–19]. We therefore hypothesized that necroptosis also occurs in TMJOA. To investigate this hypothesis, we utilized the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) and the specific necroptosis selective inhibitor Necrostatin-1 (Nec-1) in both an established *in vivo* animal model of compressive mechanical force loading and an *in vitro* model of induced chondrocyte necroptosis. As expected, both inhibitors repressed mechanical force-induced mandibular chondrocyte apoptosis and necroptosis respectively, and consequently, partially restored the cartilage thinning induced by compressive mechanical force. Furthermore, combinatorial treatment produced synergistic effects on chondrocyte survival and cartilage thickness restoration. Taken together, these data revealed a novel mandibular chondrocyte death mechanism in mandibular cartilage under compressive mechanical stress.

Materials and methods

Animals

This study was authorized by the Animal Research Committee of Nanjing University Medical School (2013NL-094) and abided by the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines for preclinical animal studies. A total of 240 (the power calculated by PASS 11.0 software was more than 80%) 7-week-old male Sprague–Dawley (SD) rats were requisitioned in this study. The rats were randomly divided into two groups: non-mechanical force (non-F) ($n = 120$) and mechanical force (F) groups ($n = 120$; 4-day and 7-day treatments), with gender- and age-matched controls according to our previous study[5] (Appendix Fig. 1).

On the first day of the experiment, 80 g of compressive mechanical force was exerted on all rats in the F group as described previously[5]. A rubber band tied between a jig and anchorage hooks was used to create the compressive mechanical force applied to the TMJ. The loading appliance was worn for 4 or 7 days. None of the rats displayed signs of disability. After compressive mechanical loading was applied for 24 or 96 h in the 4- or 7-day groups, Nec-1 and/or Z-VAD (both from Selleck, USA) were injected locally into the TMJ on one side (N: Nec-1; Z: Z-VAD; NZ: Nec-1 + Z-VAD; F N: mechanical force + Nec-1; F Z: mechanical force + Z-VAD; F NZ: mechanical force + Nec-1 + Z-VAD), and a vehicle (dimethyl sulfoxide; Solarbio) was injected on the other side, as described previously[5]. Each rat was injected once throughout the entire experiment.

Histological observation and histomorphometric measurements

After 4 or 7 days, the rats were sacrificed by cervical dislocation under anesthesia. The condyles were harvested with the surrounding tissue. Then, the specimens were fixed in 4% formalin for 24 h and decalcified in 15% ethylenediaminetetraacetic acid (EDTA) solution for 8 weeks. After a thorough rinsing, the specimens were embedded in paraffin. Next, 5-μm-thick sagittal sections were cut from each embedded TMJ block parallel to the lateral surface of the condylar neck of the mandible ramus. The paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Condylar thickness and mandibular chondrocyte number were measured on three hematoxylin and eosin (HE)-stained sections per joint[20], and the average values were used for the statistical analyses. Images were captured with an Olympus XI 70 microscope equipped with an Olympus Magna Fire digital camera. Three areas were chosen in the mandibular condylar cartilage. Thickness measurement and cell count were determined using a computer-assisted image analysis system (Image-Pro Plus, version 6.0; MediaCybernetics) at the same staining threshold. The thresholds for color balance, brightness, and texture were adjusted to best optimize all images to be compared. The measurement procedures were performed twice. The mean of the two measurements was used for further statistical analysis ($n = 12$).

Immunohistochemical staining

Immunohistochemistry was conducted as described previously[4,5]. After deparaffinization and rehydration as above, the specimens were treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity, and digested the antigenic sites with Antigen Retrieval Solution (Wuhan Boster Biological Technology Ltd. China) for 10 min. Then, the sections were incubated overnight with anti-RIP1 (1:100, Novusbio, UK), anti-RIP3 (1:300, Abcam, USA), or anti-Caspase-8 (1:100, Abcam, USA) at 4°C. Next, the specimens were incubated with biotin-labeled IgG (BeiJing ZhongShan Golden Bridge Biotechnology Co., China) for 30 min at 37°C followed by an avidin-peroxidase complex for 30 min at 37°C. A peroxidase/diaminobenzidine (DAB) yellow kit (Wuhan Boster Biological Technology Ltd., China) was used for antibody staining. The specimens were counterstained with hematoxylin, dehydrated in an ethanol series, cleared in xylene and coverslipped. Images were acquired as above for the HE-stained sections. The scores were assessed as the staining intensity (0, negative, no staining; 1, faint yellow, mild staining; 2, claybank, moderate staining; 3, brown, intense staining) and the proportion of positive cells showing cytoplasmic staining (0, <10%; 1, 10–25%; 2, 25–50%; 3, 50–75%; 4, 75–100%) ($n = 12$).

Transmission electron microscopy (TEM)

Small tissue blocks (1 mm^3) of mandibular condylar cartilage from each group were obtained and fixed in a solution containing 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 24 h, then decalcified in 15% EDTA solution for 10 days. The specimens were cut into 1-mm^3 sections and fixed in 2% osmium tetroxide (OsO_4) in 0.1 mol/L cacodylate buffer (pH 7.4) for 2 h. Next, the specimens were dehydrated in a graded alcohol series and embedded in Epon epoxy resin. Ultra-thin (75 nm) sections were obtained (LEICA ULTRACUT R) and studied without staining using a Tecnai transmission electron microscope (FEI) ($n = 5$).

Mandibular chondrocyte culture

Mandibular condylar cartilage was isolated from 3-wk-old male SD rats and subsequently minced, as described previously[5]. After the cartilage was digested with 0.25% trypsin for 30 min and with 0.2% collagenase for another 120 min, primary mandibular chondrocytes were prepared as a single-cell suspension at a density of 1×10^5 cells/cm^2 in a humidified 5% CO_2 atmosphere at 37°C. The medium was replaced every 2 days. The cells were cultured to the third generation for use in the experiments.

The cultured primary mandibular chondrocytes were divided into five groups: control (CON), TNF-α+CHX (TC, cells treated with 10 ng/L TNF-α (Sigma, US) and 0.2 mM cycloheximide (CHX, Cell

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