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Necrostatin-1 ameliorates adjuvant arthritis rat articular chondrocyte injury via inhibiting ASIC1a-mediated necroptosis

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ABSTRACT

Necroptosis, a necrotic cell death pathway regulated by receptor interacting protein (RIP) 1 and 3, plays a key role in pathophysiological processes, including rheumatoid arthritis (RA). However, whether necroptosis is involved in RA articular cartilage damage processes remain unclear. The aim of present study was to investigate the dynamic changes in arthritic chondrocyte necroptosis and the effect of RIP1 inhibitor necrostatin-1 (Nec-1) and acid-sensing ion channels (ASICs) inhibitor amiloride on arthritic cartilage injury and acid-induced chondrocyte necroptosis. Our results demonstrated that the expression of RIP1, RIP3 and mixed lineage kinase domain-like protein phosphorylation (p-MLKL) were increased in adjuvant arthritis (AA) rat articular cartilage *in vivo* and acid-induced chondrocytes *in vitro*. High co-expression of ASIC1a and RIP1 showed in AA rat articular cartilage. Moreover, Nec-1 and amiloride could reduce articular cartilage damage and necroinflammation in AA rats. In addition, acid-induced increase in necroptosis markers RIP1/RIP3 were inhibited by Nec-1, ASIC1a-specific blocker psalmotoxin-1 (PcTx-1) or ASIC1a-short hairpin RNA respectively, which revealed that necroptosis is triggered in acid-induced chondrocytes and mediated by ASIC1a. These findings indicated that blocking ASIC1a-mediated chondrocyte necroptosis may provide potential therapeutic strategies for RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is a polyarthritis featured with chronic and systemic inflammatory, resulting in synovitis, articular cartilage and bone erosion, and finally evolves into joint deformity [1]. Cartilage destruction of affected joints is one of the important causes of RA. Articular cartilage injury may be due to inflicting insults, including genetic disorders, autoimmunity and inflammation [2].

Programmed cell death including autophagy, apoptosis and necroptosis, play an important role in the regulation of cell death and survival. Of note, necroptosis, a new regulated cell necrosis pathway, depends on RIP1 and 3 kinase activity. Under pathological conditions, RIP1 and RIP3 can interact to create a filamentous amyloid protein complex called necrosome, which is required for necroptosis. RIP1 plays a critical role in necroptosis via its serine/threonine kinase activity [3,4]. After necroptotic stimulation, RIP3 is phosphorylated on Ser 199. Interestingly, Nec-1, a specific inhibitor of RIP1 kinase, could reduce RIP3 phosphorylation by inhibiting RIP1 kinase, thereby inhibiting the development of necrotic apoptosis [5]. It is well known that necrosis has a critical role in inflammatory diseases, while the molecular mechanism remains unclear [6]. Recently, it has been showed that leptin protected rat articular chondrocytes from necroptosis [7], revealing that necroptosis is partially involved in chondrocyte death. However, the role and potential mechanism of Nec-1 in RA articular chondrocyte injury are largely unknown.

Acid-sensing ion channels (ASICs) belong to the epithelial sodium channel/degenerin family, which were transiently activated by the extracellular H^+ [8,9]. ASIC1a, an important member of ASICs, plays a key role in various central nervous system diseases, including ischemic stroke, pain, and learning [10]. ASIC1a activated

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by extracellular low pH exerts pathophysiological functions by mediating calcium overload in the central and peripheral nervous systems. Tissue acidification is a common pathological feature in most pathological conditions such as inflammation, hypoxia and ischemia [11]. Interestingly, pH value reduction was also detected in synovial fluid from patients with RA and adjuvant arthritis (AA) model [12,13]. Our previous studies also indicated that ASIC1a was expressed in rat articular chondrocytes and increased in AA rat articular chondrocytes, and inhibition of ASIC1a by amiloride could inhibit articular cartilage damage [14–16]. Recently, one study has been demonstrated that ASIC1a-mediated RIP1 activation contributed to ischemic neuronal injury, and ASIC1a gene knockout significantly prevented RIP1 phosphorylation and brain injury [17]. However, the role and exact mechanism of ASIC1a in articular cartilage damage have not been fully characterized.

Accordingly, we investigated the effects of Nec-1 on ASIC1a-mediated necroptosis in AA rat articular chondrocyte. We demonstrate that Nec-1 ameliorates adjuvant arthritis rat articular chondrocyte injury via inhibiting ASIC1a-mediated necroptosis.

2. Materials and methods

2.1. Animals, grouping and treatments

140–160 g male Sprague-Dawley (SD) rats (from the Center for Laboratory Animal Sciences, Anhui Medical University, Hefei, China) were used in this study. The rats got access to water and rodent food under standard laboratory conditions at a 22 ± 3 °C temperature with a light/dark cycle (12 h/12 h). After 7 days of adaptation, the rats were randomly subdivided into the following groups: in different period's groups, non-arthritic group ($n = 8$) and AA group ($n = 40$). Then, rats in the AA group were divided respectively into 5 sub-groups by the time points: 7, 14, 21, 28, 35 days ($n = 8$). In treatment groups, non-arthritic group ($n = 8$) and AA group ($n = 24$). The latter were divided into the following groups (each $n = 8$): untreated AA group; amiloride-treated AA group; Nec-1-treated group. The experimental procedures were approved by the Ethical Regulations for the Care and Use of Laboratory Animals of Anhui Medical University, which carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. AA model was performed by injection 0.1 ml aliquot of complete adjuvant (CFA, Chondrex Inc., Redmond WA, USA) on the intraplantar subcutaneously. Intraperitoneal injection of amiloride (10 mg/kg/day) [15] and Nec-1 (1.65 mg/kg/day) [18] was begun in day 10 after immunization and continued to day 16. A control group was considered as the day 0 group, which did not receive the drug injection.

2.2. Sample harvesting and preparation

During the experiment, all of rats were weighed, rats secondary side hind paw volume was measured before inflammation and after inflammation respectively and calculated their hind paw swelling ($\triangle$ml = post-inflammatory volume - pre-inflammatory volume). Blood samples were harvested by inferior arteries under sodium pentobarbital anesthesia for ELISA experiments (day 35). Next, the animals were sacrificed and the knee joints were dissected for extraction of articular cartilage for western blot analysis; the ankle joints were fixed in 4% paraformaldehyde for 24 h and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) 5 weeks for routine histology and immunofluorescence analysis. The extracted cartilage particles were fixed in 2.5% glutaraldehyde for 24 h at 4 °C for transmission electron microscopy (TEM; JEM-1230, Tokyo, Japan) analysis.

2.3. Western blotting

The articular cartilage was comminuted into granules in a mortar, and then homogenized in a radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Samples were determined by using 10% SDS-PAGE with mouse monoclonal antibodies against RIP1, RIP3, TNF-α, IL-1β, IL-6, PGAM5 (Santa Cruz Biotechnology, USA), rabbit polyclonal anti-ASIC1a, anti-collagen type II (Abcam, Cambridge, USA) and rabbit polyclonal antibodies against p-MLKL at a dilution of 1:600, anti-β-actin at a dilution of 1:1000. The secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies. The target protein bands were obtained by an ECL-chemiluminescent kit. Quantify protein expression with an Image-Pro Plus image.

2.4. Histomorphometric analysis

The trimmed ankle joint was fixed in 4% paraformaldehyde for 24 h, and then the samples were dehydrated in an increased concentration of ethanol, finally the samples were embedded in paraffin for slice using a microtome. The undried calcified slices standard frontal sections were used for hematoxylin-eosin (HE) staining and immunohistochemical analysis. The immunohistochemical sections were incubated with 1:300 rabbit anti-rat collagen type II, 1:500 mouse anti-rat RIP1, and 1:500 mouse anti-rat RIP3. Experiment was performed as described previously [18]. Finally, the integrated optical density value (IOD) was calculated in discontinuous high-power fields under the same background light via an Image-Pro Plus image.

2.5. Double immunofluorescence staining for ASIC1a and RIP1

The sections were treated as above describe and then incubated with 1:500 rabbit anti-ASIC1a and 1:500 mouse anti-RIP1 overnight at 4 °C. After that the sections were incubated with goat anti-rabbit IgG and goat anti-mouse IgG and counterstained using DAPI; the sections were imaged using an inverted fluorescence microscope (Olympus). ASIC1a was indicated by green fluorescence, and RIP1was indicated as red fluorescence.

2.6. Transmission electron microscopy

The fixed samples from the extracted cartilage particles (1 mm^3) were demineralized in 10% EDTA for 2 weeks at 37 °C. After that the sections were fixed in 2% osmium tetroxide for 1 h, and then the sections were blocked with 2% uranyl acetate. After treatment, the cartilage particles were embedded in epoxy resin and dehydrated, the samples were cut into sections (80 nm). Finally, the sections were stained with uranyl acetate and lead citrate for observation via TEM.

2.7. Cell culture and treatment

Extraction and culture of primary rat articular chondrocytes was conducted as described previously [14]. For establishment of acidic stimulation, the cell culture medium pH was adjusted via adding HCl to achieve different pH values [19]. The primary rat articular chondrocytes were plated with a density of 2×10^4 cells per well, and then pretreated with PcTx-1 (100 ng/ml; Abcam, Cambridge, USA) for 1 h [20] or Nec-1 (30 μmol/l; Santa Cruz, USA) [21] in serum-free medium before inhibition experiment, after that chondrocytes were stimulated in the pH 6.0 solution for 2 h.

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