Simultaneously blocking necrosis and apoptosis to protect TMJ chondrocytes from TNF-alpha induced death: a preliminary study

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Abstract: Purpose: To study the effect of protecting temporomandibular joint chondrocyte from TNF-α induced death by simultaneously blocking necrosis and apoptosis in hope to find a potential adjuvant therapy target for temporomandibular joint post-traumatic osteoarthritis. Methods: Primary human condylar chondrocytes were cultured in vitro and cell growth curve was tested by CCK-8 method. Since acute post-traumatic inflammation and high levels of cytokines especially TNF-α could induce chondrocyte apoptosis, 20 ng/mL TNF-α was added to induce cell death. Before the application of TNF-α, chondrocytes were pre-treated with pan-caspase inhibitor Z-VAD-FMK (ZT Group), or specific programmed necrosis inhibitors Nec-1 (NT group), or the combination of these two inhibitors (ZNT group) or neither (T group). Apoptotic and necrotic cell death rates were detected using flow cytometry. CM-H2DCFDA staining was used to determine Reactive Oxygen Species (ROS) levels. Cell death rates and ROS levels were respectively compared and statistically analyzed. Results: T group showed exacerbated cell death and increased ROS levels in the remaining living cells, while cell death rates of ZT, NT and ZNT groups were significantly alleviated compared to T group (P<0.05). Amongst these three groups, ZNT group achieved the minimum cell death rates and ROS levels. Conclusion: Under the circumstances of a high TNF-α concentration, condylar cartilage cells could go through both apoptosis and necroptosis. Blocking both forms of programmed cell death pathways can significantly improve cell survival rate. TNF-α initiated apoptosis and necroptosis pathways might be a potential adjuvant therapy target for temporomandibular joint post-traumatic osteoarthritis.

Keywords: Temporomandibular joint, post-traumatic osteoarthritis, TNF-alpha, apoptosis, necroptosis, condylar chondrocytes

Introduction

Temporomandibular joint (TMJ) post-traumatic osteoarthritis (PTOA) is an articular degenerative disease caused by trauma to the mandible, which is pathologically manifested as a rapidly progressive loss of articular cartilage [1]. The predominant clinical symptoms of TMJ PTOA include constant pre-auricular pain and limited mouth opening. Studies have shown that chondrocyte death, extracellular matrix degradation and synovial inflammation are the main etiologic factors leading to PTOA [2]. Nevertheless, current treatments for PTOA are mainly medical treatment using drugs to relieve symptoms or condylar resection with joint reconstruction surgery for advanced end stage PTOA. Therefore, in order to develop effective therapeutic strategies for early surgical and pharmacologic interventions in the early phase after trauma, preventing chondrocyte death remains one of the most challenging issues to discuss in the present PTOA study.

Previous studies showed that there were mainly two types of chondrocyte cell death after joint trauma: direct chondrocyte necrosis caused by trauma impact [3, 4] and secondary cell death through apoptotic mechanisms in the sub-acute and chronic post-traumatic phases [5]. TMJ internal derangement often accompanies with a dramatic increased TNF-α level in the...
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synovial fluid [6-8]. Apoptosis could be induced by intraarticular inflammatory cytokines such as TNF-α [9] and executed by a series of intracellular cysteine proteases, called caspase. However, prevention of apoptosis with caspase inhibitors does not completely block cartilage cell death. Z-VAD-FMK is a non-selective, cell permeable inhibitor of broad-spectrum caspases (a family of cysteine proteases that can be activated to mediate apoptotic cell death) that was developed by MP biochemicals. A study showed that the application of Z-VAD-FMK in a rabbit knee joint cavity reduced the size of cartilage lesion and increased chondrocyte number by 31%, but it did not significantly affect the OA lesion grade compared with the control group [10]. Latest research revealed that when the caspase activity was inhibited, TNF-α pathway could shift from apoptosis to another kind of programmed cell death-necroptosis [11] through the interaction with receptor interacting protein 1 (RIP1) and RIP3 [12], which assembled into an amyloid scaffold to recruit other proteins to form high molecular weight complexes, called necrosomes [13]. Necrostatin-1 (Nec-1) is a small molecule that specifically inhibits the phosphorylation activity of RIP1 (receptor interacting protein 1), therefore blocks the programmed necrosis pathway. Necroptosis has been involved in several pathological processes, including viral infection [14, 15], myocardial ischemia-reperfusion [16], brain ischemic injury [11] and multidrug resistance of tumor cells [17, 18], whether it plays a role in PTOA has not been reported yet.

This study was designed to study the effect of protecting TMJ chondrocyte from TNF-alpha induced death by simultaneously blocking necrosis and apoptosis in hope to increase chondrocyte survival rate after trauma, which may serve as a potential adjuvant therapy for TMJ PTOA.

Materials and methods

Chondrocytes isolation and in-vitro cultivation

Condylar cartilage fragments were obtained from patients undergoing open reduction and internal fixation surgery of traumatic TMJ injury. The samples were out of plan to be used in reconstruction of the condyle and would otherwise have been discarded. All patients gave their written consent to participate in the study. This study was conducted according to the guidelines in the Declaration of Helsinki and all procedures involving human subjects were approved by the Human Ethics Committee of Shanghai Jiao Tong University Affiliated Ninth People’s Hospital. Samples were kept at 4°C in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) supplemented with 100 I.U. of penicillin G. and 100 mg/ml of streptomycin (Sigma-Aldrich, USA) and processed within the following 24 h.

Mechanical separation combined with sequential enzymatic digestion method was used to isolate the chondrocytes from the fragments. The cartilage was diced with a scalpel and digested in a shaker at 37°C for 30 min with 1% NB-4 collagenase (SERVA 17454.01, Germany) in high glucose DMEM. Then the isolated cells were collected and centrifuged at 1200 rpm, 5 min. New 1% NB-4 collagenase was added and the procedure was repeated till the fragments were fully digested. Cell viability was detected by trypan blue methods [19], 90% to 95% of the recovered cells were alive. The isolated cells were washed, concentrated and seeded into a flask to be cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium used was high glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 I.U. penicillin G. and 100 mg/ml streptomycin. Subconfluent cells were passaged with 0.25% trypsin-0.02% EDTA (Gibco, USA) and subcultured in five consecutive passages (P1-P5). P2 chondrocytes were further seeded on 14 mm diameter round cover glass (WHB, China) in 24-well plates to perform toluidine blue and type II collagen staining. Primary chondrocytes showed characteristics consistent with cartilage cell identification. P3-P5 chondrocytes were used for subsequent experimental steps.

Toluidine blue staining

P2 cells were seeded and cultured on 14 mm diameter round cover glass in 24-well plates. Wash the cells with cold phosphate buffered saline (PBS) 3 times, 3 min each time. Cells were then fixed with 4% 1 mL paraformaldehyde for 20 min at room temperature. Parafomaldehyde was washed off with deionized water and the cover glasses were placed on a slide. Then 1% toluidine blue liquid was added and incubated at room temperature for 30 min
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in the dark. Then the dye was removed with deionized water. Cover glasses were dehydrated through gradient alcohol and xylene and then mounting.

Type II collagen staining
P2 cells were cultured on round cover glasses and fixed as above. Then 3% H$_2$O$_2$ were added and incubated for 10 min at room temperature in order to reduce the nonspecific background staining caused by endogenous peroxidase. After cells were washed 3 times with PBS, 10% goat serum blocking solution were added and incubated for 10 min at room temperature. Drain the blocking serum and no rinse. Then mouse anti-human type II collagen antibody was added to completely cover the round glass, and incubated at 37°C for an hour, followed by the incubation of HPR goat anti-mouse antibody. Negative control cover glass cells received PBS incubation instead of mouse anti-human type II collagen antibody with other procedures identical to the other. DAKO Real Envision Detection Kit (DAKO, USA) was applied as instructed.

Growth curve
The effect of primary chondrocytes proliferation was assessed using the cell counting kit (CCK-8; Dojindo, Kumamoto, Japan). Generally, chondrocytes were suspended and seeded in 96-well plates with 2×10$^3$ cells/well and then cultured for 7 d. CCK-8 solution was added to 6 wells each day at intervals of 24 hours followed by incubation for another 4 h at 37°C according to the reagent instructions, the absorbance at 450 nm was measured using an ELISA microplate reader (Tecan Sunrise, Switzerland). Cell viability was directly proportional to the absorbance at 450 nm. The experiment was done in triplicate.

Treatments
P3-P5 chondrocytes were seeded into 6-well plates at a starting density of 1×10$^6$ cells/well. After being attached to the plate bottom and became flattened, the chondrocytes were cultured overnight (16 hours approximately) with a serum-deprived medium. Prior to TNF-α stimulation, the chondrocytes were pre-treated with apoptosis inhibitor Z-VAD-FMK, necroptosis inhibitor Nec-1, or both Z-VAD-FMK and Nec-1 for 30 min. Cells could be divided into 4 groups (Table 1): TNF-α group (T group), apoptosis inhibition group (ZT group), necrosis inhibition group (NT group), and apoptosis and necrosis simultaneously inhibition group (ZNT group). There is a dose-dependent effect of TNF-α in cell proliferation and death, while 20 ng/mL has been proved to be the optimal concentration [8]. So T group contained 20 ng/mL TNF-α and 0.1% DMSO (solvent for Z-VAD-FMK and Nec-1), ZT group contained 20 uM Z-VAD-FMK and 20 ng/mL TNF-α, NT group contained 10 uM Nec-1 and 20 ng/mL TNF-α, ZNT group contained 20 uM Z-VAD-FMK, 10 uM Nec-1 and 20 ng/mL TNF-α.

Cell survival rate
P3-P5 chondrocytes were seeded into 96-well plates at a concentration of 3×10$^3$ cells/well. Then cells were treated for 4 h as described above with 6 replicate wells per group. CCK-8 solution was added and the cells were incubated for another 4 h before the absorbance at 450 nm was measured.

Apoptosis detection via flow cytometry
For assessment of apoptosis, chondrocytes were labeled with PI (Propidium Iodide) and Annexin V FITC using the Annexin V FITC Apoptosis Detection KIT (BD 556547, USA), according to the reagents instructions. The 4 groups of chondrocytes were treated for 8 h as described above, then cells including the dead floating cells in the supernatant were collected and washed with cold PBS 3 times and centrifuged at 1200 rpm, 5 min and then adjusted to a concentration of 1×10$^6$ cells/ml with a binding buffer. For each group of cell suspension, 5 uL of Annexin V-FITC and 5 uL of PI working solution were added and incubated for 15 min at 4°C. Untreated chondrocytes were used as a double-negative control. Untreated chondrocytes dyed with Annexin V-FITC or PI served as a single negative control. Chondrocyte apoptosis and necrosis were detected by Flow

Table 1. Condylar chondrocytes treatments

<table>
<thead>
<tr>
<th>Groups</th>
<th>T group</th>
<th>ZT group</th>
<th>NT group</th>
<th>ZNT group</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>20 ng/mL</td>
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<tr>
<td>Z-VAD-FMK</td>
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<td>Nec-1</td>
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ROS levels

CM-H$_2$DCFDA is a commonly used general ROS indicator [20]. After the chondrocytes were treated for 7.5 as described above, 10 uM CM-H$_2$DCFDA was added to the culture medium and incubation was maintained at 37°C for 30 min, then mixed gently every 5 min, giving a great care to avoid light exposure. At the end of the treatment period, the supernatant was aspirated and the adherent cells were washed 3 times with warm PBS to remove the excess CM-H$_2$DCFDA, then cells were collected and flow cytometry was used to detect fluorescence at an excitation wave length of 488 nm and emission wave length of 525 nm within 30 min.

Statistical analysis

Data were expressed as the mean ± standard deviation of triplicate or more values for each experiment. Statistical analysis was performed using SPSS15.0 software package and GraphPad Prism. One-way analysis of variance (ANOVA) and $P$ value was used to compare multiple groups of chondrocyte proliferation, apoptosis and necrosis, and ROS levels. $P<0.05$ was considered significant.

Results

Primary human condylar cartilage cells were polygonal with several long processes, with round or oval nuclei and heavily populated cells area showed a cobble stone-like appearance (Figure 1A). Type II collagen staining showed brown positive staining areas in the cytoplasm (Figure 1B). Toluidine blue staining showed the cells were dyed blue, with variable amounts of purple metachromatic granules in the cytoplasm (Figure 1C).

The expansion capacity of chondrocytes exhibited an obvious improvement in apoptosis and
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The chondrocyte apoptosis level (the upper right and the lower right quadrants) of ZT group was lower than that of T group (P<0.05, Figure 3A, 3B). Nec-1 alone did not reduce the level of apoptosis, but ZNT group achieved the lowest apoptosis level (Figure 3E). Necrosis levels (the upper left quadrant) of ZT, NT, and ZNT groups were lower than T group. Although apoptosis of ZT group chondrocytes was inhibited, there was still a small part of cells continued through necrosis. ZNT group had a lower level of necrosis than both ZT and NT groups (P<0.01, Figure 3F).

Similarly, ROS levels in the remaining living cells of ZT, NT and ZNT groups were all lower than T group, furthermore, ZNT group ROS level was significantly less than ZT group (P<0.001, Figure 4E), indicating that the combination of Z-VAD-FMK and Nec-1 could reduce cell ROS level more effectively.

Discussion

Acute trauma to the TMJ is commonly seen clinically, and it is often prone to progress into PTOA. It has been estimated that in 9% to 85% of patients, condylar fractures result in a degenerative disease of the TMJ [21]. TMJ PTOA patients could present with chronic pain with or without radiation, limited mouth opening, crepitus, TMJ clicking, popping, locking, deviation on mouth opening, and even facial asymmetry with occlusal disorders. Treatments for PTOA are currently limited to drugs (mainly non-steroidal anti-inflammatory drugs, sulfated glycosaminoglycans, hyaluronic acid, etc.) to relieve symptoms or artificial joint replacement surgery for an end stage PTOA [22]. How to intervene effectively in the early phase after trauma to prevent, slow down or reverse the development of PTOA, remains an urgent clinical need to meet.

Previous studies showed that acute post-traumatic inflammation and high levels of cytokines TNF-α could induce chondrocyte apoptosis. But patients on TNF-α inhibitors (infliximab, adalimumab, etanercept, and certolizumab) for treatment of juvenile idiopathic arthritis, ankylosing spondylitis or inflammatory bowel disease (IBD), could be at a higher risk of developing fatal leukemia and severe autoimmune diseases [23]. So TNF-α inhibitors may not be specific enough for PTOA.

However, prevention of apoptosis with caspase inhibitors could not completely block cartilage cell death and decrease OA lesion grade [10].

Figure 2. Chondrocytes growth curve and proliferation. The chondrocytes expansion capacity of apoptosis and necrosis simultaneously inhibition group (ZNT group) exhibited an obvious improvement whereas a slow proliferation capacity was detected with TNF-α treated cultures (T group). Cell viability of ZNT group at 6 h and 8 h culture period was more promising than T group and ZT group (Figure 2B).

A. P3 chondrocytes growth curve. B. The proliferation ability of P3 chondrocytes assessed using CCK-8 analysis. Error bars indicate the standard deviation of the mean; *P<0.05, **P<0.01 by Student’s t test.
Latest research revealed that when caspase activity was inhibited, TNF-α pathway could shift from apoptosis to another kind of programmed cell death, namely necroptosis. Although necroptosis has been involved in certain cell types and some diseases [24-26], however, whether it occurs in PTOA has not yet been studied. The specific mechanism of TNF-α...
Necrosis and apoptosis of TMJ chondrocytes remained unclear. So in order to demonstrate the chondrocyte death manners in TNF-α signaling pathway and the effect of protecting TMJ chondrocyte from TNF-alpha induced death by simultaneously blocking necrosis and apoptosis, we built this in-vitro primary condylar cell model to conduct a preliminary study on chondrocyte TNF-α pathway.

TNF-α pathway activation could potentially lead to three different cell destinies as studies shown [27]. Stimulation of TNFR1 by TNF-α leads to the formation of intracellular complex I at the cytoplasmic membrane that include TRADD, TRAF2, RIP1, and cIAP1, which would further activate NF-κB pathway. If RIP1, FADD and caspase-8 instead form into complex IIA, then the caspase-mediated apoptosis pathway will be activated. When caspase activity was inhibited, RIP1, RIP3 and some macromolecules assembled into complex IIB and lead to cell necroptosis. Nec-1 blocks the necroptosis pathway by specifically inhibiting the kinase activity of RIP1. So theoretically we need to block apoptosis and necroptosis simultaneously in order to achieve a highest cell survival rate.

ROS is considered to be one of the executors of necrosis by oxidating intracellular proteins or

Figure 4. ROS levels detected by CM-H2DCFDA staining method. ROS levels in the remaining living cells of ZT, NT and ZNT groups were all lower than T group. FL1-H peak shifted to the left and decreased Mean M1 value means the decrease of ROS level. A. T group. B. ZT group. C. NT group. D. ZNT group. E. ROS levels of 4 groups. Error bars indicate the standard deviation of the mean; ***P<0.001 by Student’s t test.
Necrosis and apoptosis of TMJ chondrocytes interfering with other downstream pathways such as prolongation of the activation of JNK pathway [28, 29]. We used CM-H$_2$DCFDA (6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), as a ROS indicator, and flow cytometry to detect DCF fluorescence to reflect the intracellular ROS levels during chondrocytes necrosis [20]. Due to the ROS increase during necroptosis and Nec-1’s specific necroptosis inhibition ability, we used these two indicators to detect the occurrence of necroptosis indirectly.

In this study, chondrocytes exhibited a decreased apoptosis rate with the application of Z-VAD-FMK, but we didn't find an exacerbated cell death as He et al. [12] observed in their experiments using Smac mimetic, TNF-α, and Z-VAD-FMK to induce necroptosis in human colon cancer HT-29 cells. We suspected that possibly most of the chondrocyte cells did not shift to necroptosis but survived through the NF-κB pathway. With the survival rate of the ZNT group significantly higher than ZT and NT groups plus the survival rate of NT group higher than T group, it indicated that a part of chondrocytes death was executed through necrosis. However, the main mode of chondrocyte death manners under TNF-α stimulation was still apoptosis. Chondrocyte necroptosis could be accompanied with an increase in ROS levels as it showed in our experiment.

Still, our preliminary study requires in-depth research of chondrocyte necroptosis mechanisms both in-vitro and in-vivo. Animal studies of the effect of protecting TMJ chondrocytes from TNF-alpha or trauma induced cell death by blocking necrosis and apoptosis simultaneously needs to be conducted additionally.

In conclusion, under circumstances of a high TNF-α concentration, condylar cartilage cells could go through both apoptosis and necroptosis. Inhibiting both forms of programmed cell death pathways can significantly improve cell survival rate. TNF-α initiated apoptosis and necroptosis pathways might be a potential adjuvant therapy target for TMJ PTOA.

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**Disclosure of conflict of interest**

None.

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