

Increase of TNF α -stimulated Osteoarthritic Chondrocytes Apoptosis and Decrease of Matrix Metalloproteinases 9 by NF- κ B Inhibition

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Abstract

Objective To investigate the in vitro effect of caffeic acid phenethyl ester (CAPE), a NF- κ B inhibitor, on the apoptosis of osteoarthritic (OA) chondrocytes and on the regulation of the gelatinases matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9).

Methods Annexin V-FITC/propidium iodide (PI) labeling and western blotting were used to observe and determine the apoptosis in TNF α -stimulated primary cultured osteoarthritic chondrocytes. Also, gelatin zymography was applied to examine MMP-2 and MMP-9 activities in supernatants.

Results It was confirmed by both flow cytometry and western blotting that chondrocytes from OA patients have an apoptotic background. Use of CAPE in combination with 10 ng/mL of TNF α for 24 h facilitated the apoptosis. MMP-9 in the supernatant could be autoactivated (from proMMP-9 to active MMP-9), and the physiologic calcium concentration (2.5 mmol/L) could delay the autoactivation of MMP-9. The activities of MMP-2 and MMP-9 in the fresh supernatant increased significantly in response to stimulation by 10 ng/mL of TNF α for 24 h. The stimulatory effect of TNF α just on proMMP-9 was counteracted significantly by CAPE.

Conclusion NF- κ B could prevent chondrocytes apoptosis though its activation was attributed to the increase of proMMP-9 activity induced by TNF α (a pro-apoptotic factor). Therefore, therapeutic NF- κ B inhibitor was a 'double-edged swords' to the apoptosis of chondrocytes and the secretion of MMP-9.

Key words: Chondrocytes; Gelatinase; Apoptosis; NF- κ B; Tumor necrosis factor α

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INTRODUCTION

Osteoarthritis (OA) is the most widespread cause of physical morbidity which impairs the quality of life all over the world. And nowadays, OA continues to burden

Chinese society that population is under aging^[1].

Several previous studies on impact of Tumor Necrosis Factor (TNF α), an important inflammatory factor, on the onset and progression of OA reported that concentration of TNF α in OA synovial fluid^[2] and even in the serum and subchondral bone was higher

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than normal controls^[3]. There were also some other studies focusing inhibition of TNF α resulting in slowing down the disease progression. The inhibition of TNF α indeed yielded some benefits on relieving articular swelling and inhibiting cartilage damage triggered by TNF α in rat model^[4]. Besides inflammation, degradation of articular cartilage is also a major feature of OA. And as we know, extracellular matrix (ECM) is very important to maintain normal cartilage. Disrupted balance between ECM synthesis and degradation, in favor of proteolysis, leads to pathologic cartilage destruction. Matrix metalloproteinases (MMP) belongs to a family of 23 enzymes which facilitate ECM breakdown^[5]. MMP-2 and MMP-9, which are gelatinases, are correlated with osteoarthritis development. Increased gene expressions of MMP-2 and MMP-9 were detected in OA patients^[6] and significant increased concentration of MMP-9 were found in synovial fluids of joints of the patients with severe cartilage alterations^[7]. Their secretion could also be elevated by TNF α ^[8]. Therefore, we believe that there should be some association between inflammatory factors and gelatinases in OA.

Nuclear factor-kappa B (NF- κ B), while inducing inflammation^[9-11], is also implicated in protection of the osteoarthritic chondrocytes^[12-13], playing a role as 'double-edged swords'. It also played an important role in TNF α induced MMP-9 expression in human tracheal smooth muscle cells^[14] and adenocarcinoma cells^[15].

So far, little is known about whether the inhibition of NF- κ B could affect chondrocytes secreted gelatinases, especially when studying the fate of chondrocytes at the same time. Therefore, we observed the effect of CAPE on OA chondrocytes apoptosis induced by TNF α and the activity of MMP-2 and MMP-9 in this study.

MATERIALS AND METHODS

Reagents

CAPE (NF- κ B inhibitor) was from Sigma-Aldrich; Dulbecco's Modified Eagle's Medium (DMEM) and FBS were from Gibco; 4%-12% Bis-Tris Nu-PAGE gels were from Invitrogen. Gelatin and Triton X-100 were from Amresco. Annexin Calbiochem Annexin V-FITC Apoptosis Detection Kit was from Merck, Germany.

Primary Culture of OA Patients' Chondrocytes

Cartilage samples were collected from the femoral condyles of 3 female patients with knee OA

(mean age is 65.3 with a range of 63-67). None of the patients had a clinical history of traumatic arthritis or any other pathological changes affecting the cartilage. The cartilage specimens were obtained from the patients who underwent total knee arthroplasty at Peking Union Medical College Hospital (PUMCH) and met the American College of Rheumatology criteria for OA. The study was approved by the Ethical Committee of Clinical Investigation of PUMCH. And the written informed consents were obtained from all patients prior to the study. Pieces of articular cartilage were cut, minced, and incubated with collagenase type II (Sigma, St. Louis, catalog number 17 101-015) in DMEM until the fragments were digested. Released cells were spun, washed, and seeded at 2×10^6 /plate in 6-well tissue culture plates in DMEM supplemented with 10% FBS, 1% L-glutamine, and penicillin/streptomycin (150 U/mL and 50 mg/mL each) in 5% CO₂ at 37 °C. After about 7-10 days, confluent chondrocytes were split once, seeded at 1×10^5 /well in 6-well culture plates, and these first passage chondrocytes were used in the subsequent experiments.

Induction and Inhibition of Apoptosis

To elucidate the effect of TNF α on OA cell viability, 5×10^4 cells/well was seeded in 12-well culture plates. Before adding TNF α , the medium containing 10% FBS was replaced with DMEM containing 0.5% FBS, and the chondrocytes were incubated overnight. After 24 h, the cells were treated with 1 μ mol/L CAPE for 1 h before adding 10 ng/mL of TNF α . Cells were harvested after 24 h of TNF α treatment for immunoblotting analysis, and supernatants of the cultured chondrocytes were harvested for analysis of MMP-2 and MMP-9 by gelatin zymography.

Quantification and Verification of Apoptosis

In order to study apoptosis, flow cytometry was applied using annexin V-FITC/propidium iodide (PI) labeling and quantification of cleaved caspase-3 and PARP was conducted by western blotting. For flow cytometry, floating and adherent cells were collected, washed, and resuspended in cold binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂) to a final concentration of 1×10^6 /mL. Aliquots of 5×10^5 cells were incubated with 1.25 μ L of annexin V-FITC (200 μ g/mL) for 15 min at room temperature. Then, the media was removed and the cells were gently resuspend in 0.5 mL cold binding buffer and 10 μ L of PI (30 μ g/mL)

was added afterwards. Samples were placed on ice and kept away from light before flow cytometric analysis was conducted. For each sample, at least 10^4 cells were analyzed on a FACS II flow cytometer (Beckman Coulter Inc., USA). FITC and PI fluorescences were passed through 520 nm and 630 nm bandpass filters, respectively. Anti-cleaved PARP (1:2000) and anti-cleaved caspase-3 (1:3000) were used as the primary antibodies for the western blotting analysis and the details of the methodology was described in our previous report^[12].

Gelatin Zymography

In order to analyze the stability of MMP-2 and MMP-9, we stored each of the aliquot of supernatant mixed with different concentrations of calcium (2.5 mmol/L, 200 μ mol/L, or 10 μ mol/L) at -80 °C. Before sampling, the protein concentrations were determined by using a BCA Protein Assay Kit (Pierce Biotechnology, USA, Rockford, IL) with bovine serum albumin as a standard. For gelatin zymography, after mixing with an equal volume of nonreducing 2 \times Laemmli sample buffer, 40 μ g total protein of supernatants were loaded per lane and electrophoresed at 15 mA on 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing copolymerized gelatin (1 mg/mL). Afterwards, zymograms were washed 5 times in 2.5% Triton X-100 (20 min each) and 2 times in buffer without Triton X-100 to remove Triton X-100, and then incubated in 50 mmol/L Tris-Cl, pH 7.6 and 5 mmol/L CaCl₂ (18 h, 37 °C) in a thermostatically controlled water bath. Zymograms were stained with 0.2% Coomassie blue R-250 in 50% methanol and 10% acetic acid and destained appropriately. MMP-2 and MMP-9 activities, identified as 'cleared' (i.e., degraded) regions against a dark background, were compared to known gelatinase molecular weight standards. After reversion between the dark and light areas, BandScan 5.0 software was used to quantitate total gray that is parallel to the protein activity.

Western Blotting

Cells were lysed in lysis buffer (2% SDS, 10% glycerol, 10 mmol/L Tris, pH 6.8, 100 mmol/L DTT), and then subjected to immunoblotting as described previously. Before sampling, the protein concentrations were measured by using a BCA Protein Assay Kit (Pierce Biotechnology, USA, Rockford, IL) with bovine serum albumin as a standard. Samples were then combined with gel loading buffer (50 mmol/L Tris-Cl, pH 6.8, 2% SDS,

10% glycerol, and 0.1% bromphenol blue), boiled for 5 min, and electrophoresed on 12% SDS-PAGE gel for anti-cleaved caspase-3, 8% SDS-PAGE gel for anti-cleaved PARP. Proteins were wet-blotted onto polyvinylidene difluoride (PVDF) transfer membranes, and blots were blocked with Tris-buffered saline (TBS) containing 5% non-fat milk for 1 h, and incubated with anti-cleaved PARP (1:2000), anti-cleaved caspase-3 (1:3000) for 3 h at room temperature. The blots were then rinsed and incubated with 1:5000 HRP-conjugated IgG goat anti-mouse or rabbit anti-goat Ab for 1 h. The blots were then washed and developed by use of a Super Enhanced chemiluminescence detection kit (Applygen Technologies Inc., Beijing, China) and the protein bands were visualized after exposure of the membranes to Kodak film (USA).

Statistical Analysis

Results are represented as the mean \pm SEM. All in vitro experiments were repeated at least 3 times with cultured primary cartilage chondrocytes for each treatment. Differences between mean values were compared by Student's *t*-test for unpaired observations using GraphPad Prism software (GraphPad Software, San Diego, CA), assuming normal distribution where 3 replicates were used. *P* values less than 0.05 were considered significant.

RESULTS

Induction of Chondrocytes Apoptosis with TNF α and CAPE

As shown in Figure 1, the OA chondrocytes expressed cleaved caspase-3 and PARP, indicating that OA chondrocytes have apoptotic background. Treatment of chondrocytes with 10 ng/mL of TNF α alone could not produce any significant change of cell death after 24 h of incubation revealed by the unchanged expression levels of either cleaved caspase-3 or PARP via western blotting (Figure 1) and flow cytometry (Figure 2A and 2B). In order to investigate whether NF- κ B is related with TNF α -induced chondrocytes apoptosis, a dual apoptosis mediation experiments was conducted using CAPE. Co-treatment with NF- κ B inhibitor CAPE (Figure 1) and TNF α increased the expressions of cleaved caspase-3 and PARP and produced more apoptosis chondrocytes. Flow cytometry also showed the similar result that more FITC (+) chondrocytes (3.4%, Figure 2C) in the TNF α and

CAPE co-treatment group were found compared to those (2.4%, Figure 2A) in control group.

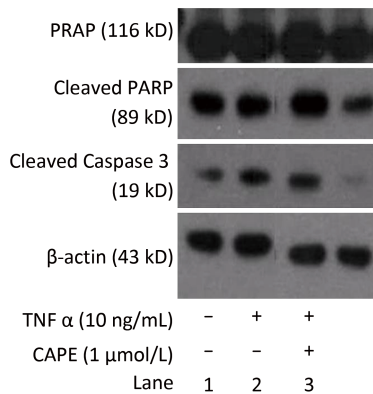


Figure 1. The expressions of cleaved PARP and caspase-3 in OA chondrocytes stimulated with recombinant human TNF α at 10 ng/mL for 24 h by western blotting, with β -actin as normalization. Lane 1: OA chondrocytes without any stimulation; Lane 2: OA chondrocytes stimulated with recombinant human TNF α at 10 ng/mL for 24 h; Lane 3: OA chondrocytes stimulated with recombinant human TNF α at 10 ng/mL and CAPE at 1 μ mol/L for 24 h; Lane 4: OA chondrocytes stimulated with CAPE at 1 μ mol/L for 24 h (representative of 3 independent experiments).

Stability of MMP-9 in Different Concentrations of Calcium

Owing that proMMP-9 could undergo autoactivation to lower molecular weight forms (active MMP-9) in the synovial fluid of rheumatoid arthritis (RA) patients and could be delayed by presence of physiologic calcium concentration^[16], we tried in this study to study the proper supernatant collecting time and storage temperature for future experiments. In the fresh supernatant after stimulation of chondrocytes for 24 h, the activity of active MMP-9 just covered 15.6% of the total activities of MMP-9. While, as shown in Figure 3, at the 6th day and without calcium in the supernatant, the ratio of activity of active MMP-9 in the total activities of MMP-9 increased to nearly 90%. Also, until the 9th day, it increased up to nearly 100% and among the three different concentrations of calcium (2.5 mmol/L, 200 μ mol/L, and 10 μ mol/L), the physiologic calcium concentration of 2.5 mmol/L could mostly delay the autoactivation of MMP-9.

Therefore, from the aspect of MMP-9 stability, we collected the fresh supernatant of calcium free for the further gelatin zymography analysis.

Effects of TNF α and NF- κ B Inhibitor on The Activities of MMP-2 and 9

As shown in Figure 4, 10 ng/mL of TNF α significantly increased the activities of all forms of MMP-9 and MMP-2, including proMMP-9, active MMP-9, proMMP-2 and active MMP-2. While, NF- κ B inhibitor CAPE plus TNF α significantly decreased the activity of proMMP-9 compared to the TNF α stimulation group and there were no significant changes in their activities by CAPE stimulation alone.

DISCUSSION

We examined the change of apoptosis rate of OA chondrocytes cultured in 10 ng/mL of TNF α and NF- κ B inhibitor CAPE and also investigated the changes of activity of gelatinases secreted in the supernatants in this study. It was found that there were high levels of basal cleaved caspase-3 and PARP in OA chondrocytes and this was consistent with the data from flow cytometry. 10 ng/mL of TNF α could not cause any significant change of apoptosis, while NF- κ B inhibitor CAPE could cause more apoptosis in the stimulation of TNF α . Therefore, OA chondrocytes have an apoptotic background and NF- κ B had anti-apoptotic effect. Our result was consistent with those reported by Relic et al. that both NF- κ B inhibitor MG-132 and adenovirus-expressing mutated I κ B α could induce more chondrocytes apoptosis stimulated by TNF α and no donor sodium-nitro-prusside (SNP)^[17]. It is therefore important for researchers to pay more attention to the chondrocytes apoptosis while lowering down the NF- κ B activities.

Several previous studies have found that gelatinases were associated with osteoarthritis. Kim et al. examined gelatinases MMP-2 and MMP-9 and the collagenases MMP-1 and MMP-13 in arthritic joint and found that MMP-9 and MMP-13 may play a more important role in angiogenesis in RA and OA^[18]. Hulejova et al. found that in contrast to control samples, OA cartilage and synovium showed significantly higher MMP-2, -3, -9 and up-regulated MMP-9 in OA subchondral bone^[3]. Still other studies also supported some associations between gelatinases and osteoarthritis^[19-24].

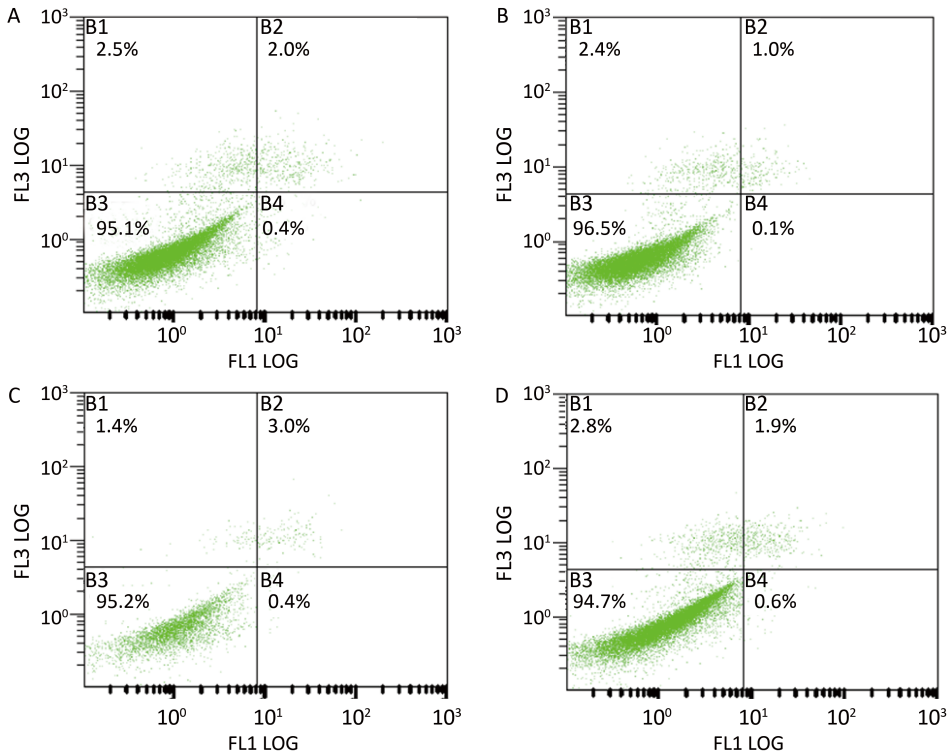


Figure 2. Flow cytometry analysis of annexin V-FITC/propidium iodide (PI) dual staining of primary chondrocytes from OA patients. A: medium only; B: human recombinant TNF α (10 ng/mL), and after 24 h, the cells were stained as described in the text; C: TNF α (10 ng/mL) + CAPE (1 μ mol/L); D: CAPE (1 μ mol/L). X-axis and Y-axis indicate FITC negative/positive and PI negative/positive, respectively. Numbers in right upper and right lower quadrant (indicated in Figure 2A) denote percentages of late and early apoptotic chondrocytes (Annexin V positive).

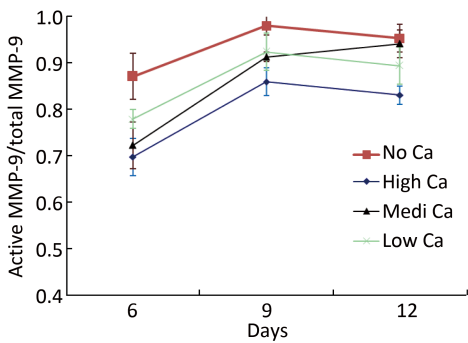


Figure 3. The ratio of changes of active MMP-9 among the total MMP-9 (Y-axis showed) by means of gel zymography in serial days after collecting the fresh supernatants (X-axis showed) in the supernatants of chondrocytes from OA patients which were stored at -80 $^{\circ}$ C and with different concentrations of calcium (high concentration: 2.5 mmol/L, medium concentration: 200 μ mol/L and low concentration: 10 μ mol/L) as shown in colorful lines.

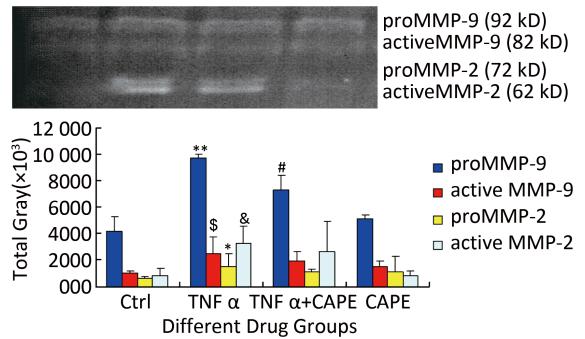


Figure 4. Human OA chondrocytes were cultured with or without stimulation with TNF α (10 ng/mL) or CAPE (1 μ mol/L) for 24 h. The MMP activity in the conditioned medium is identified on the zymography gel by the standards for MMP-2 and MMP-9. Data represents the mean \pm SEM of the total gray values of different MMP-2/9 in the supernatants of chondrocytes from three OA patients under different stimulations of drugs. SEM=standard error of the mean. **proMMP-9: $P < 0.001$ vs. ctrl; # $P < 0.01$ vs. TNF α ; \$active MMP-9: $P < 0.05$ vs. ctrl; #proMMP-2: $P < 0.05$ vs. ctrl; &active MMP-2: $P < 0.05$ vs. ctrl.

We found in our present study the relative higher activities of proMMP-9 and active MMP-9 than MMP-2 in the control group of osteoarthritic chondrocytes. We believe that it might be due to much inflammatory reactions in the osteoarthritic chondrocytes of osteoarthritis patients. Gregory et al. showed that proMMP-9 in synovial fluid of osteoarthritis patients was associated with the level of white blood cell. Well in the contrast, proMMP-2 was present in all synovial fluids regardless white blood cell count. Activated synovial fluid MMPs persisted despite resolution of infection^[25]. The similar phenomenon was also present in synovial fluid from calves of patients with experimentally induced septic arthritis^[26]. Therefore, proMMP-9 may be more relevant to inflammatory process. However, MMP-2 was found to be a general chemokine antagonist which dampens inflammatory reaction, through monocyte chemoattractant protein-3 (MCP-3) cleavage^[27-28]. Therefore and to some extent, higher activities of MMP-9 than MMP-2 represent the unbalance of more inflammatory factors than anti-inflammatory ones.

High concentration of TNF α was found in synovial fluid of osteoarthritis patients^[2] and might be an important factor to the onset and development of OA^[3-4]. We found that 10 ng/mL of TNF α could significantly increase the activities of both forms of MMP-2 and -9. In a previous study, both 10 ng/mL of IL-1 β and TNF α significantly enhanced expression of MMP-9 and NF- κ B p65-specific siRNA could reduce the enhancement at the levels of mRNA transcription and protein synthesis^[29]. However, the researchers of this study did not further examine MMP-2 and figure out whether proMMP-9 or active MMP-9 was more involved. And gelatin zymography offered a reliable and specific method to analyze pro- and activated gelatinases, suggesting some posttranslational modifications of the pro-gelatinases^[30-31].

Through Gelatin zymography, we also found that NF- κ B inhibition could significantly reduce the increased proMMP-9 activity by TNF α , but could not produce any change in activities of active MMP-9, proMMP-2 and MMP-2 and this was similar to those reported by Xue et al. Treatment of mature monocytes cell line Mac6 with 6-AQ, a specific inhibitor of NF- κ B activation, inhibited TNF α -stimulated MMP-9 production in a dose-dependent manner, but has no effect on MMP-2^[20]. Constitutive activation of NF- κ B has been reported in RA and OA patients, and was known to

be central for the regulation of the synthesis and activity of inflammatory cytokines^[32]. As we mentioned above, proMMP-9 may be more relevant to inflammatory process and it is therefore concluded that inhibition of NF- κ B is beneficial to lower down the inflammation of osteoarthritic chondrocytes.

In summary, NF- κ B inhibition raised a controversial on the cell apoptosis and gelatinases secretion in osteoarthritic chondrocytes. NF- κ B inhibition caused more chondrocytes apoptosis induced by a pro-apoptotic factor TNF α . However, the same degree of its inhibition decreased the activity of proMMP-9. Therefore, we suggest that it should be cautious to keep the balance while taking NF- κ B as a therapeutical target.

It should be mentioned that there are limitations of this paper. For example, NF- κ B inhibition selectively act upon MMP-9 without affecting MMP-2. This is interesting and it suggest a differential transcriptional control of enzymes with similar activities. Also, it would be interesting to see these effects at the level of mRNA transcription and future studies at molecular level are needed to verify the results in experimental animals.

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