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Taurochenodeoxycholic acid induces apoptosis of fibroblast-like synoviocytes

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ABSTRACT

Recent evidences have suggested that the paucity of the apoptosis of fibroblast-like synoviocytes (FLS) may contribute to the pathogenesis of rheumatoid arthritis. Apoptosis induction of rheumatoid arthritis FLS is therefore suggested as a potential therapeutic approach for rheumatoid arthritis. Taurochenodeoxycholic acid (TCDCA), one of the main bioactive substances of animals' bile acid, could favorably ameliorate the progression development and bone destruction of adjuvant arthritis in rat. In this study, we aimed to investigate the possible effect of TCDCA on apoptosis induction of adjuvant arthritis FLS and the mechanisms involved in this process. Apoptosis was determined by flow cytometric analysis. Gene expression levels and the activities of caspase-3 and caspase-8 were evaluated using real time RT-PCR and luminogenic substrates. The activity of nuclear factor-κB (NF-κB) was measured by ELISA. The results showed TCDCA significantly enhanced the apoptosis of adjuvant arthritis FLS in a dose-dependent manner. Besides, TCDCA treatment markedly increased the gene expression level and activity of both caspase-3 and caspase-8. It could suppress the DNA-biding activity of NF-κB. We concluded TCDCA represented an apoptotic effect on adjuvant arthritis FLS via the activation of caspase cascade and this process may be mediated by NF-κB signaling pathway. It was suggested that TCDCA may be a potential therapeutic agent for rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis is a chronic autoimmune disease associated with multiple inflammatory mediators that lead to joint damage, hyperplasia of synovial tissue, inflammatory infiltrates, and a progressive destruction of cartilage and bone (Firestein, 2003; Ono et al., 2004). The prevalence of rheumatoid arthritis varies worldwide between 0.5 and 1% (Brenner et al., 2005). Although various cell populations may participate in the pathogenesis of rheumatoid arthritis, fibroblast-like synoviocytes (FLS) are considered crucial in both the initiation and progression of arthritis (Huber et al., 2006). The mechanisms of synovial hyperplasia are not fully understood, although the paucity of the apoptosis may contribute to the pathogenesis of rheumatoid arthritis. Recent evidence suggests that the activation of FLS in rheumatoid arthritis is associated with a reduced level of apoptosis in vivo, especially at sites of invasion into cartilage and bone (Baier et al., 2003). Apoptosis induction of rheumatoid arthritis FLS is therefore suggested as a potential therapeutic approach for rheumatoid arthritis (Ospelt et al., 2004; Pope, 2002).

Adjuvant arthritis is a rodent model of experimental arthritis that is useful tools to study the pathogenic process of rheumatoid arthritis (Kannan et al., 2005). Adjuvant arthritis is induced by injection of Freund's complete adjuvant (FCA). The similarities between the joint pathologies as well as the cellular and humoral immunities in adjuvant arthritis and rheumatoid arthritis suggest that adjuvant arthritis is a relevant animal model that acts as a useful test system for evaluating apoptosis-inducing therapies (Bendele et al., 1999; Huang et al., 2008).

Bile acids are synthesized in the liver from oxidation of cholesterol and stored in the gallbladder as the main constituent of bile. Chenodeoxycholic acid (CDCA) and cholic acid (CA) are the two primary bile acids and are conjugated mainly to glycine (G) and taurine (T), which are taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and taurocholic acid (TCA) (Hofmann, 1984). It is clear that bile acids not only facilitate digestion, absorption, and excretion of dietary lipids, but also interact with numerous cellular signaling pathways (Argmann et al., 2006; Bouscarel et al., 1999; Hirano et al., 2006; Nguyen and Bouscarel, 2008). TCDCA is synthesized with taurine and chenodeoxychlolic acid in organism, which is one of principal bioactive substances of bile from animal bile. In our previous study, TCDCA conferred remarkably inhibition on both acute and chronic inflammation, especially it favorably ameliorate the progression development and bone destruction of adjuvant arthritis in rat (Liu et al., 2011a), which suggests that TCDCA may

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be a potential therapeutic agent for rheumatoid arthritis. However, it remains unclear whether TCDCA directly influences FLS functions such as apoptosis. The aim of the current study was to determine whether TCDCA could induce FLS apoptosis in adjuvant arthritis rats. The potential mechanisms were also discussed.

2. Materials and methods

2.1. Reagents

Freund's complete adjuvant (FCA) (Shanghai institute of biological products, China), Caspase-Glo® 3/7 Assay, Caspase-Glo® 8 Assay (Promega, USA), FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA), Tripre™ RNA regent, M-MulV Reverse Transcripts and SYBR® Premix Ex Tag™ (TaKaRa Biotechnology, China), Nuclear Extract Kit and TransAM® NF-κBp65 kit (Active Motif, USA).

2.2. TCDCA dissociated and depurated

Fresh chicken galls were collected from slaughterhouse. Bile was deproteinated using alcohol after filtered by filter paper, and then it was condensed using rotary evaporator after depigmented by activated carbon. Crude bile acids were obtained after saltingout, extracting and dewatering. TCDCA was dissociated and depurated from crude bile acid by chromotography techniques and the purity was detected by high performance liquid chromatography and its purity was $>99.5\%$.

2.3. Animals and induction of adjuvant arthritis rat

The study was approved by Institute's Animal Ethical Committee and confirmed to national guidelines on the care and use of laboratory animals. Male wistar rats, 11–13 weeks old, weighing 160–180 g, were obtained from experimental animal center, academy of military medical sciences in China. All animals were maintained at a controlled temperature (22 ± 2 °C), and a regular light/dark cycle (7:00–19:00, light), and all animals had free access to food and water. Adjuvant arthritis rat was induced as previously described (Huang et al., 2008; Kim et al., 2004). Briefly, rats were immunized on day 0 by intradermal injection of FCA into the foot pad, containing 10 mg heat-inactive Bacillus Calmette-Guerin in 1 mL paraffin oil, into the left hind paw in 0.1 mL for each rat.

2.4. Isolation and culture of adjuvant arthritis FLS

Adjuvant arthritis FLS were isolated as previously described with modifying (Huang et al., 2008). Briefly, fresh synovial tissues were obtained from adjuvant arthritis rats under sterile conditions. The synovium was minced with fine scissors, incubated in a plastic flask (Corning, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10 mM HEPES (pH 7.2) (Promega, USA), 20% fetal calf serum (FCS) (TBD, China), 100 U/ml penicillin and streptomycin (Gibco, USA) 50 mM mercaptoethanol in a humidified 5% CO_2-containing atmosphere at 37 °C for 7 days. After removal of the synovial pieces, the adherent cells were cultured in the same medium. At 70–80% confluence, nonadherent cells were removed, and adherent cells were trypsinized, split at a 1:3 ratio and recultured in the same medium. The synoviocytes were used in experiments from passages 3. After three passages, most of the cultured synoviocytes comprised a homogeneous population of FLS.

2.5. Flow cytometry

FLS were obtained from adjuvant arthritis rats were incubated in DMEM containing TCDCA (50 mg/ml, 100 mg/ml, 200 mg/ml, 400 mg/ml) for 48 h, while control FLS were incubated in DMEM alone. Then, the cells were trypsinized and collected for detection of apoptosis with FITC Annexin V Apoptosis Detection Kit I according to the manufacturer's protocol. Briefly, the cells were washed twice with cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Then, 100 μl of the solution (1×10^5 cells) was transferred to a 5 ml culture tube and 5 μl of FITC-Annexin V was added following addition 5 μl of propidium iodide (PI). After incubation for 15 min at room temperature in the dark, samples were immediately analyzed using FAC-SCalibur flow cytometer (BD Biosciences, USA). Approximately 1×10^4 cells were collected and analyzed with CELLQuest software (BD Biosciences, USA).

2.6. RNA isolation and real time RT-PCR

Total FLS RNA was extracted using Tripre™ RNA regent and both the ratio of $OD_{260/280}$ and agarose gel electrophoresis were generated to ensure the quality of RNA. Synthesis of cDNA was performed using an M-MulV Reverse Transcriptase with random 9-mers according to the manufacturer's protocol. cDNA was amplified using SYBR®Premix Ex Tag™ (Perfect Real time) kit with specific oligonucleotide primers for target sequences and in a total of 25 μl reaction mixture (2 μl of cDNA, 12.5 μl of 2 × SYBR®Premix Ex Tag™, 1 μl of each 10 μM forward and reverse primers and 8.5 μl of H_2O). The real-time quantitative PCR thermal cycling conditions were 95 °C for 30 s, followed by 95 °C for 5 s and Tm for 30 s for 35 cycles. The following primers were used: *β-actin* forward: 5′-GGA GAT TAC TGC CCT GGC TCC TA-3′ and reverse: 5′-GAC TCA TCG TAC TCC TGC TTG CTG-3′, *caspase-3* forward: 5′-GAG ACA GAC AGT GGA ACT GAC GAT G-3′ and reverse: 5′-GGC GCA AAG TGA CTG GAT GA-3′ and *caspase-8* forward: 5′-TCA GCA ACA TGC GGG ACA G-3′ and reverse: 5′-TGA AGC AGT CTT TGC CCT TGT G-3′. All primers were synthesized by TaKaRa Biotechnology (China). Data were analyzed according to the comparative Ct method (Liu et al., 2011b) and were normalized by *β-actin* expression in each sample. Relative mRNA levels were calculated based on the Ct values, corrected for *β-actin* expression, according to the equation: $2^{-\Delta Ct}$ [ΔCt=Ct (*caspase-3 or caspase-8*) –Ct (*β-actin*)]. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

2.7. Assays for caspase-3 and caspase-8 activities

Caspase-3 and caspase-8 activities were measured using Caspase-Glo® 3/7 assay kit and Caspase-Glo® 8 assay kit. All procedures were carried out according to the manufacturer's instructions. Briefly, adjuvant arthritis FLS were seeded into a white 96-well plate (Nunc, USA) at a density of 5000 cells/well in triplicate wells. Cells were cultured in the presence or absence of TCDCA for 48 h and subsequently mixed with an equal volume of caspase substrates. Luminescence was measured after 30 min incubation using a plate reader (Synergy 4, Bio-tek, USA).

2.8. DNA-binding activity of NF-κB p65

Specific binding of NF-κBp65 subunit was measured using a TransAM® NF-κBp65 kit according to the manufacturer's instructions. After treatment with TCDCA, cells were collected by scraping and centrifugation. Cell nuclear extracts were then added into a 96-well plate immobilized with oligonucleotide containing the NF-κB consensus site (5′-GGG ACT TTC C-3′). Using a primary antibody directed against the NF-κBp65 subunit and a second antibody conjugated to horseradish peroxidase, the optical density