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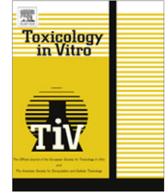

Effects of T-2 toxin and selenium on chondrocyte expression of matrix metalloproteinases (MMP-1, MMP-13), α_2-macroglobulin (α_2M) and TIMPs

Jinghong Chen, Yonglie Chu, Junling Cao *, Wei Wang, Jiayuan Liu, Jiali Wang

Institute of Endemic Diseases, Medical School of Xi'an Jiaotong University, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Xi'an 710061, Shaanxi, PR China

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ABSTRACT

T-2 toxin is regarded as an important etiological factor of Kashin-Beck disease, and supplementation of selenium–salt partly prevents Kashin-Beck disease. The present study investigated the effects of T-2 toxin on the degradation of type II collagen in human chondrocytes *in vitro*. Human chondrocytes were isolated and cultured on bone matrix gelatin to form an artificial cartilage model *in vitro* with or without T-2 toxin and selenium. Immunohistochemistry analyses showed that T-2 toxin decreased type II collagen staining and selenium appeared to prevent the decrease in type II collagen induced by T-2 toxin in engineered cartilage. Then, Western blot and RT-PCR analyses showed that an increase in MMP-13 and MMP-1 expressions, and a decrease in the expression of the general endoproteinase inhibitor (α_2M) were induced by T-2 toxin. Gelatin reverse zymography showed that TIMP-1 and TIMP-2 levels were decreased in a dose-dependent manner after exposure of T-2 toxin. Selenium had a protective role by increasing the level of type II collagen protein through down-regulation of MMP-13 protein and mRNA expression and up-regulation of TIMP-1 and TIMP-2 expressions. These data suggest T-2 toxin induces cartilage matrix degradation by the up-regulation of MMP-13 and TIMP-1, and down-regulation of TIMP-2 and α_2M expressions.

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1. Introduction

Kashin-Beck disease is a chronic, deforming endemic osteoarticular disease of unknown etiology. The pathological features of Kashin-Beck disease are the degeneration and death of chondrocytes in the deep layer of cartilage and a chaotic metabolism of the cartilage matrix (Allander, 1994). T-2 toxin contamination in food in endemic area probably contributes to the pathogenesis of Kashin-Beck disease in humans (Yang, 1995; Yao et al., 2010). One previous study from chick model showed that administration of T-2 toxin can lead to some pathologic changes in articular cartilage of chicks, which is similar to changes in Kashin-Beck disease (Yang, 1995). Recently, a study from rat model found that T-2 toxin combined with a low-nutrition diet can lead to more serious chondrocyte necrosis in the epiphyseal plate and disturb metaphyseal trabecular bone formation (Yao et al., 2010). In addition, apoptosis of chondrocytes can be induced by T-2 toxin *in vitro* (Chen et al., 2006).

The molecular mechanisms underlying cartilage destruction in Kashin-Beck disease are poorly understood. Cartilage is made up of two main extracellular matrix (ECM) macromolecules: type II collagen and aggrecan, a large aggregating proteoglycan. The type II collagen endows the cartilage with its tensile strength, whereas aggrecan enables cartilage to resist compression. Other minor components of the cartilage (e.g. type IX, XI and VI collagens, biglycan, decorin and cartilage oligomeric matrix protein) also have important roles in controlling the supramolecular organization of the matrix (Eyre, 2002). Normal cartilage ECM is in a state of dynamic equilibrium, with a balance between synthesis and degradation. Cartilage turnover is regulated by a balance between proteinases that degrade the ECM and their inhibitors. In Kashin-Beck disease, a disruption of this balance, in favor of proteolysis, leads to pathological cartilage destruction (Cao et al., 2008).

Cartilage destruction is thought to be mediated by two main enzyme families: the matrix metalloproteinases (MMPs) and enzymes from a distintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS). The MMPs are responsible for cartilage collagen breakdown, whereas enzymes from ADAMTS family mediate cartilage aggrecan loss. Tissue inhibitors of

Abbreviations: ECM, extracellular matrix; BMG, bone matrix gelatin; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; α_2M, α_2-macroglobulin; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; DME/F12, Dulbecco's Modified Eagle's medium-F12; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, trisbuffered saline.

* Corresponding author. Tel.: +86 13 087580882; fax: +86 29 82655032.
E-mail address: caojl@mail.xjtu.edu.cn (J. Cao).

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metalloproteinases (TIMPs) are endogenous inhibitors of MMPs and are also potential inhibitor of the ADAMTS family. The ability of the TIMPs to block active MMPs is largely promiscuous (Murphy et al., 2002).

MMPs are a family of structurally related zinc-dependent neutral endopeptidases, which can be classified into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs. When activated, MMPs degrade a broad spectrum of substrates, including collagens and other matrix macromolecules. On the whole, MMPs play an important role in the extracellular matrix remodeling that occurs under physiological and pathological conditions (Nagase and Woessner, 1999).

α_2-Macroglobulin (α_2M) is a high molecular weight proteinase inhibitor, which is abundantly present in body fluids and also in synovial fluid during inflammation (Beekman et al., 1997). It is suggested that α_2M plays an important role in the inhibition of activated MMPs in body fluids (Cawston and Mercer, 1986). Active MMP is expected to be captured by the proteinase inhibitor α_2M to form an α_2M/MMP complex capable of inactivating the MMPs (Grinnell et al., 1998). Moreover, α_2M has been shown to be a novel substrate for ADAMTS-4 and ASAMTS-5 that are responsible for the breakdown of aggrecan, and represents an endogenous inhibitor of these enzymes (Tortorella et al., 2004).

T-2 toxin is one of the mycotoxins, a group of type A trichothecenes produced by several fungal genera including Fusarium species. T-2 toxin is detected in a number of field crops (wheat, maize, barley and oats) and processed grains (malt, beer and bread). T-2 toxin has been shown to cause a variety of toxic effects in both experimental animals and humans. In the present study, we showed that T-2 toxin induced a dose- and time-dependent inhibition of the cellular proliferation of human chondrocytes. We evaluated the role of T-2 toxin in human chondrocytes, focusing on type II collagen as well as the relevant major enzymes, MMP-1, MMP-13 and their inhibitors TIMP-1, TIMP-2 and α_2M.

2. Materials and methods

2.1. Chemicals

Crystallized trypsin, collagenase type II, chondroitinase ABC, testicular hyaluronidase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). T-2 toxin was kindly provided by Professor Jinsheng Yang, Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences. Mouse CP18 anti-collagen II, was purchased from Merck4 Bioscience (Darmstadt, Germany). Rabbit anti-MMP-1 and MMP-13 antibodies were from Chemicon International, Inc. (Temecula, CA, USA). Rabbit anti-α_2M was from R&D Systems (Minneapolis, MN, USA). Anti-rabbit IgG was from Jackson ImmunoResearch Laboratories Inc. (Bar Harbor, ME, USA). Rabbit anti-β-actin antibody and Goat anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's Modified Eagle's medium-F12 (DME/F12) and Trizol reagent were purchased from Gibco (Grand Island, NY, USA). SABC kits from Boster Co. (Wuhan, China). RT-PCR kits were products of Fermentas Life Sciences (Lithuania). Super Signal Ultra Western blot chemiluminescence system was purchased from Pierce Life Science (Rockford, IL, USA). All other chemicals were of the highest grade available from commercial sources.

2.2. Chondrocyte isolation and cultures with T-2 toxin

Articular cartilage was obtained as previously described (Chen et al., 2006). The study was approved by Animal Ethics Committee, Xi'an Jiaotong University, School of Medicine. Slices of cartilage were aseptically dissected and chondrocytes were obtained by sequential digestion with hyaluronidase, trypsin and collagenase type II as previously described (Wei et al., 1987). Cells were cultured to confluence at 37 °C in a humidified atmosphere containing 5% CO_2, in complete medium (DME/F12 with 15% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin). All experiments were performed using first passage of chondrocytes to avoid any de-differentiation.

Primary chondrocytes were grown for 24 h after the initial passage, then the DME/F12 medium was replaced by the same medium containing various concentrations (0, 1, 10, 20 ng/mL) of T-2 toxin and/or sodium selenite (final concentration of selenium 0.1 μg/mL). Cultures were incubated for 5 days at 37 °C and 5% CO_2. The selenium concentration chosen was based on the normal range in serum (0.065–0.33 μg/mL) in the Chinese population (Xia, 2004).

2.3. Engineered cartilage preparation

Human fetal chondrocytes were isolated and seeded on bone matrix gelatin (BMG) scaffolds for 24 h, and continuously cultured for 14 days in the absence or presence of T-2 toxin at 10 ng/mL with or without Se supplementation at 0.1 μg/mL (Mark et al., 1978). The BMG grafts were prepared as described preciously (Li et al., 2006). BMG scaffolds were sterilized with ethylene oxide and pre-incubated in DME/F-12 with 15% FBS for 1 h at 37 °C. The BMG scaffolds were pelleted down at the bottom of 15 mL Falcon centrifuge tubes (Mark et al., 1978). Chondrocytes (5.0×10^5) were then seeded on the BMG graft. The reconstructed cartilage was cultured for 14 days *in vitro* with medium changing every other days.

2.4. Cell viability assay by MTT

Cells (5×10^3) were seeded into individual 96-well plates. After 24 h of culture, complete medium (DME/F-12 with 15% fetal calf serum) with or without T-2 toxin (1–8000 ng/mL) was added and incubated for different time (1–5 days). Then, 10 μL MTT at a final concentration of 500 μg/mL was added into the culture medium. After 4 h, the medium containing MTT was aspirated and replaced by DMSO for 0.5 h. The optical density (OD) was then measured in ELISA plate reader at 490 nm.

2.5. Immunolocalization of type II collagen, MMPs

Engineered cartilage was fixed using paraformaldehyde. The paraffin-embedded cartilage samples were cut into 6-μm sections and placed on poly-L-lysine-coated glass slides, which prevents detachment of sections. Sections were deparaffinized and endogenous peroxidase activity was deactivated using 3% H_2O_2/100% MeOH for 15 min at room temperature and rinsed several times in trisbuffered saline (TBS). For staining MMP-13 and MMP-1, sections were treated with 0.25% trypsin dissolved in TBS with a pH of 7.6 for 15 min at 37 °C. A final wash with TBS was done to stop all enzyme activities. Type II collagen was stained following treatment with 0.25% trypsin, sections were then treated with testicular hyaluronidase at 1.45 IU/mL for 15 min and chondroitinase ABC at 0.25 IU/mL for 15 min for antigen unmasking. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. After blocked with 10% normal serum of the species in which the secondary antibody was produced. Primary antibodies were incubated overnight at 4 °C. After extensive washing with TBS, the appropriate biotin-labeled secondary antibody (1:1000) was used for 30 min at 37 °C, followed by a strept–avidin–biotin–peroxidase complex (SABC) method using SABC kits. Counterstaining was performed with hematoxylin. The negative controls for

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