Effect of autophagy induced by dexamethasone on senescence in chondrocytes

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Abstract. The aim of the current study was to explore the effects of dexamethasone (DXM) on autophagy and senescence in chondrocytes. Collagen II and aggrecan were examined in normal chondrocytes isolated from Sprague-Dawley rats. Following stimulation with DXM, LysoTracker Red staining, monodansylcadaverine (MDC) staining, green fluorescent protein-red fluorescent protein-light chain 3 (LC3) and western blotting were used to detect autophagy levels in the chondrocytes. Mechanistic target of rapamycin (mTOR) pathway-associated molecules were investigated by western blotting. Cell senescence was analyzed by senescence-associated (SA)-β-galactosidase (β-gal) staining. A dose-dependent increase in the number of autophagic vacuoles was observed in the DXM-treated chondrocytes, as demonstrated by LysoTracker Red and MDC staining. A dose-dependent increase in autophagosome formation was observed in the DXM-treated chondrocytes. Expression of LC3-II and beclin-1 was increased by DXM, in particular in the cells treated with DXM for 4 days. However, P62 expression was reduced as a result of treatment. SA-β-gal staining indicated that DXM increased cell senescence. Notably, DXM-induced cell senescence was exacerbated by the autophagic inhibitor 3-MA. Autophagy induced by DXM protected chondrocytes from senescence, and it is suggested that the mTOR pathway may be involved in the activation of DXM-induced autophagy.

Introduction

Osteoarthritis (OA) is a common disease, and its incidence rate in people over 25 years old is 14%, with ~400,000 patients diagnosed with OA per year in the Catalonia region of Spain (1). Clinically, local intra-articular injection of DXM has an anti-inflammatory function, relieving the symptoms of knee OA, such as pain and swelling. However, long-term and repeated injections of DXM can disrupt the metabolic balance in chondrocytes, leading to the catabolism in the cartilage with increase of matrix metalloproteinases (MMPs), and can accelerate cell apoptosis and death. This may result in a reduction in chondrocyte number and further deterioration of articular cartilage in OA. Therefore, a novel method to maintain the survival of chondrocytes under DXM stimulation is required.

Autophagy, a self-protective mechanism, has been suggested to maintain cellular homeostasis by removing protein aggregates and damaged organelles by the fusion of autophagosomes and lysosomes. Previous studies have demonstrated that autophagy is a protective process preventing against chondrocyte apoptosis and cartilage degeneration (2,3). In a mouse model, local injection of rapamycin, an inducer of autophagy, has been demonstrated to impede the progress of arthritis (2,4). It is widely accepted that autophagy in mammalian cells can be adaptively activated by different stresses (5-7), therefore, it was hypothesized that DXM may be able to activate autophagy in chondrocytes.

Previous studies have demonstrated that the ability of joint tissues to restore the articular surface is reduced with age; patients with intra-articular fractures of the knee >50 years have a greater risk of developing OA than younger patients (8,9). Age also increases the risk of post-traumatic OA in patients with anterior cruciate ligament tears (9). With the aging of cartilage, senescent chondrocytes may accumulate in the cartilage, leading to the loss of ability to maintain and restore articular cartilage effectively (10). A previous study additionally observed a clear correlation between increasing age and incidence of OA (11). Aging is the largest risk factor for OA, and cell senescence serves an important role in the occurrence and development of OA. However, the exact mechanism involved in the effect of DXM on chondrocyte senescence remains unclear.

A previous study demonstrated that autophagy is a protective mechanism in human chondrocytes with mitochondrial dysfunction (3). These observations suggest that autophagy serves an important role to protect chondrocytes from oxidative stress, and additionally supported the theory that pharmacological interventions targeting autophagy may...
prevent cartilage degradation (3). The effect of autophagy on chondrocyte senescence remains to be fully elucidated. Therefore, the current study aimed to culture rat chondrocytes treated with DXM, in order to determine the effect of DXM on chondrocyte senescence and autophagy and the association between these two events.

Materials and methods

All procedures involving Sprague-Dawley rats were performed under the approval and guidance of the Animal Care and Use Committee at Southern Medical University (Guangzhou, China).

Rat knee chondrocyte culture. A total of 34 3-month-old Sprague-Dawley male rats, ranging in weight between 300 and 340 g, were obtained from the Laboratory Animal Center of Southern Medical University. These rats were anesthetized by 10% chloral hydrate and sacrificed by cervical dislocation, and the articular cartilage was separated from the femoral condyles and tibial plateaus under a microscope. Cartilage slices were incubated with trypsin (0.5 mg/ml) (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 30 mins at 37°C. Subsequent to removal of trypsin, the cartilage slices were incubated with 0.1% collagenase, type II (Sigma-Aldrich; Merck Millipore) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% fetal calf serum (FCS; Life Technologies; Thermo Fisher Scientific, Inc.) for 4 h at 37°C with shaking. The isolated chondrocytes were recovered and plated in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. The chondrocytes were incubated at 37°C in a humidified gas mixture containing 5% CO2 balanced with air. In the current study, all cells used were second-generation chondrocytes. DXM (Sigma-Aldrich; Merck Millipore) was added to the chondrocytes at various concentrations (0, 0.1, 1, 25 and 50 µg/ml).

3-MA and DXM were added to chondrocytes at various concentrations (0 µg/ml, 10 mmol/l 3-MA, 10 mmol/l 3-MA + 25 µg/ml DXM and 25 µg/ml DXM).

Alcian blue staining. Chondrocytes were fixed with 4% paraformaldehyde for 30 min at 37°C. Chondrocytes were stained with 1% Alcian Blue 8GX (Sigma-Aldrich; Merck Millipore) dissolved in glacial acetic acid for 30 min at room temperature. The sections were counterstained with 0.1% nuclear β-galactosidase Staining kit for frozen sections (Sigma-Aldrich; Merck Millipore) for 30 min. The stained cells were washed three times with phosphate-buffered saline (PBS) and observed by light microscopy.

LysoTracker Red staining. Chondrocytes (3x10^5 cells/well) were fixed with various concentrations of DXM for 4 days at 37°C in 24-well plates and rinsed with DMEM three times. LysoTracker Red culture medium (66 mM; 1 ml) was added to each well and then the cells were cultured for 30 min at 37°C. Subsequent to washing with PBS three times, the chondrocytes were observed using an inverted fluorescence microscope.

Monodansylcadaverine (MDC) staining. Monodansylcadaverine is a specific in vivo marker for autophagocytic vacuoles. Chondrocytes (3x10^5 cells/well) were fixed at different concentrations of DXM for 4 days at 37°C in 6-well plates and rinsed with PBS three times. Subsequently, chondrocytes were incubated at 37°C in 0.05 mM MDC (Sigma-Aldrich; Merck Millipore) for 30 min. Following incubation, chondrocytes were washed three times with PBS at 37°C and fixed for 30 min in 4% paraformaldehyde. Following fixation, chondrocytes were washed four times with PBS and observed under a fluorescence microscope. To observe the rate of autophagy, stained chondrocytes were tested using flow cytometry.

Western blotting. Chondrocytes incubated with various concentrations of DXM (0, 0.1, 1, 25 and 50 µg/ml) were washed with PBS. Total proteins were isolated, and were mixed with a standard protein solution from the Bicinechonic Acid (BCA) assay kit to produce the BCA working fluid. Each well was filled with the working fluid (200 µl) for 15 min at room temperature. The protein concentration was determined according to the measured optical density values. According to the protein concentration of each group (sample quantity, 30 µg; total volume, 20 µl), the total amount of protein in each group was calculated. Subsequent to heating with loading buffer (Beyotime Institute of Biotechnology, Haimen, China) and PBS at 100°C for 5 min, protein samples were prepared. Equal quantities (30 µg) of protein from each sample were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with 5% nonfat milk, the membranes were incubated with the following rabbit anti-rat primary antibodies overnight at 4°C: Polyclonal anti-beclin-1 (1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab55878), mouse monoclonal anti-p62 (1:1,000; Abcam; cat. no. ab56416), rabbit polyclonal anti-LC3 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse polyclonal anti-β-actin (1:1,000; Beyotime Institute of Biotechnology; cat. no. AA128). Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; 1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 2 h at 37°C. The bands were detected using ECL plus reagent (Invitrogen; Thermo Fisher Scientific, Inc.) on an enhanced chemiluminescence detection system (PerkinElmer, Inc., Waltham, MA, USA). In addition, the intensity of the bands was quantified using Alpha Ease FC software, version 4.0 (Alpha Innotech Corporation; ProteinSimple, Santa Clara, CA, USA).

β-galactosidase (β-gal) staining. Chondrocytes were treated with 3-MA and DXM at various concentrations (0 µg/ml, 10 mmol/l 3-MA, 10 mmol/l 3-MA + 25 µg/ml DXM and 25 µg/ml DXM) for 24, 48 and 72 h; then, chondrocytes were stained using a Senescence β-Galactosidase Staining kit for flow cytometry. The stained cells were observed using a phase-contrast microscope to determine the percentage of positive cells out of the total chondrocytes under magnification of x200.

Red fluorescent protein (RFP)-green fluorescent protein (GFP)-light chain 3 (LC3) assay. Prior to treatment with glucocorticoids, chondrocytes were transfected with monomeric
It was observed that the intensity of MDC staining. In the control group, only certain cells were stained positive for MDC (Fig. 2A) and the intensity of MDC staining. The proportion of β-gal-positive chondrocytes significantly increased with time upon treatment with 25 µg/ml glucocorticoid, and was greatest following treatment for 72 h (Fig. 4A-E). However, the proportion of β-gal-positive chondrocytes increased significantly when 3-MA, which can inhibit autophagy, was added in comparison with the DXM group alone after 72 h. Furthermore, 3-MA alone was not able to increase the proportion of β-gal-positive chondrocytes (Fig. 4F).

Discussion

In the current study, it was investigated whether DXM is able to inhibit chondrocyte autophagy through an mTOR-dependent pathway and induce senescence. Senescence activated by DXM was observed to be inhibited by autophagy, and it was identified that chondrocyte senescence increased with the inhibition of autophagy.

DXM is widely used to relieve a variety of symptoms caused by OA, however long-term and repeated treatment often results in complications (12,13). DXM can lead to chondrocyte apoptosis, growth inhibition, and a reduction in biological activity (13,14). Apoptosis may reduce the number of chondrocytes and reduce the extracellular...
matrix, including proteoglycan and collagen type II, thus it is suggested that chondrocyte apoptosis is an important factor in the pathogenesis of OA (15). While autophagy is a programmed cell death program similar to apoptosis, the effects of glucocorticoid treatment on chondrocyte autophagy remain to be investigated.

Autophagy is not only similar to apoptosis but also has a protective effect on chondrocytes in OA (2,3,16). Autophagy has gained research focus due to its potential in regulating the aging process. A previous study has reported that the activity of autophagy was reduced in senescent tissues (17). When cells encounter certain stress situations or bacterial invasion, double membranous vesicles called autophagosomes are formed. These autophagosomes either fuse with endosomes or lysosomes, leading to the formation of autolysosomes. The process begins with the formation of a double membranous
phagophore that elongates into an autophagosome, during which cellular material becomes enclosed (18). In this way, autophagy can transport larger organelles or pathogens. 

In vitro, autophagy can inhibit chondrocyte apoptosis and prevent degradation of the extracellular matrix induced by interleukin 1β (19). In vivo, it remains unclear whether intraperitoneal injection of rapamycin or local intra-articular injection of rapamycin can promote chondrocyte autophagy and reduce the progress of degeneration of articular cartilage (2,4). In the current study, it was identified that DXM significantly activates chondrocyte autophagy, thus it was hypothesized that autophagy may be compensatory to the chondrocyte damage induced by DXM.

mTOR is one of the components of mTOR complex 1 (mTORC1) and serves as a key switch in regulating autophagy, thus the autophagic signal pathway can be divided into
mTOR-dependent and mTOR-independent pathways (20). Commonly, the stimulation of autophagy begins with the inhibition of mTOR, and the most important of two downstream targets of mTORC1 are p70S6K and 4EBP1. It was identified that DXM can reduce p70S6K and 4EBP1 phosphorylation and activity, and thus, DXM activates autophagy in chondrocytes through an mTOR-dependent pathway. Similarly, glucosamine, which is the current treatment for OA, also activates autophagy through an mTOR-dependent pathway (21). These results demonstrate that the predominant signal pathway of autophagy activation in chondrocytes is mTOR-dependent.

DXM can reduce cell growth and inhibit cell activity. Poulsen et al. (22) identified that DXM can reduce tendon cell growth and induce senescence. However, whether DXM can induce chondrocyte senescence remains to be determined.
When cell-cycle progression is arrested, the cell can be in a static state or become senescent. When the cell senesces, cell proliferation stops, however its volume loss continues to increase, resulting in aging cells being larger than normal cells (23). In the current study, it was identified that DXM can promote chondrocyte senescence and that this effect is more marked as time progresses. Chondrocyte senescence has been considered to be a correlative factor for OA, thus it is suggested that glucocorticoid-induced senescence may be an explanation for chondrocytes undergoing degeneration and necrosis.

The association between autophagy and senescence remains controversial. Kamalakannan et al (24) identified that autophagy can prevent mononuclear cell senescence caused by heat shock protein, however it induces bronchial epithelial cells to undergo senescence caused by oxidative stress (25). For chondrocytes, senescence was activated when 3-MA was used to inhibit autophagy, which indicates that glucocorticoid-induced autophagy may be a compensatory protective effect against senescence.

In conclusion, the current study identified that DXM can inhibit chondrocyte autophagy through an mTOR-dependent pathway and induce senescence. Autophagy may therefore serve as a protective process against senescence.

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