The dual role of autophagy in chondrocyte responses in the pathogenesis of articular cartilage degeneration in osteoarthritis

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Abstract. The present study aimed to analyze the responses to autophagy in osteoarthritis (OA) and aging chondrocytes in order to elucidate the role of autophagy in the pathogenesis of OA. We used multiple assays to confirm that autophagic activity was downregulated in chondrocytes of aged articular cartilage. Surprisingly, we found that the expression of autophagy-related proteins was not decreased in the tissues of patients with OA. We also observed that rapamycin-induced autophagy prevented the accumulation of subdiploid cells in young chondrocytes, while it induced cell death by autophagy in OA chondrocytes. Our results demonstrate that autophagic activity decreases with aging, and may be responsible for the cytoprotective effects in young cartilage. However, we found that autophagic activity in patients with OA was higher than in the aging group, and reported autophagic cell death in OA chondrocytes. These results suggest that autophagy plays both a cytoprotective and death-promoting role in the pathogenesis of OA.

Introduction

Osteoarthritis (OA), the most common chronic disability in older adults, is characterized by the degeneration of articular cartilage. With the aging of the population, it is estimated that the number of OA cases will double over the next 3 decades (1). Recent studies have suggested that aging plays an important role in the pathogenesis of OA (2,3). However, whether the aging process is responsible for the development of OA has not yet been fully elucidated. Considering that chondrocytes are the only type of cell type present in articular cartilage, the OA process is characterized by the changes that occur in these cells. Therefore, the understanding of the cellular processes that regulate aging-associated changes in chondrocytes is essential to the development of novel therapeutic interventions for progressive joint diseases.

Autophagy is a cellular response to various types of stress, whereby cellular organelles and macromolecules are engulfed and recycled to sustain cellular metabolism. Evidence indicates that autophagy is required for lifespan extension in various organisms, and that numerous autophagy-related proteins are directly regulated by longevity pathways (4). Autophagic dysfunction may contribute to the pathogenesis of numerous neurodegenerative diseases, including Parkinson's disease (5), Alzheimer's disease (6), Frontoteporal dementia (7), Lafora disease (8) and Huntington's disease (9). Constitutive autophagy is important for maintaining the quality of organelles and maintaining cell function. Autophagy degrades damaged organelles, cell membranes and proteins, and the failure of autophagy is thought to be one of the main causes of cell aging. Therefore, the level of autophagic activity decreases with aging, whereas the stimulation of autophagy may have potent anti-aging effects (10).

However, to date, little is known about the role of autophagy in articular cartilage. Roach *et al* (11) firstly described a peculiar variant of apoptotic cell death in articular cartilage, termed chondroptosis, a term that also included an autophagy component. Previous studies have suggested that autophagy is associated with OA. Caramés *et al* (12) demonstrated that aged and OA articular cartilage were associated with the reduced expression of Unc-51-like kinase 1 (ULK1), Beclin-1 and microtubule-associated protein 1 light chain 3 (LC3), and speculated that autophagy may play a protective role against chondrocyte death. In addition, hypoxia-inducible factor (HIF)-2α has been suggested to suppress autophagy in chondrocytes (13). Certainly, autophagy plays a dual role, and is involved in both cell survival and death (4,14). However, the potential role of autophagy in the initiation or the development of OA remains unknown. Therefore, further studies are required to elucidate the possible role of autophagy in OA and to understand the association between autophagy, cell survival and cell death.

In present study, we investigated the role of autophagy in chondrocyte responses in the pathogenesis of articular cartilage degeneration in OA. By intervening in autophagy, we assessed the differences among the chondrocytes and ultrastructural changes. Our results demonstrated that Beclin-1 and

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LC3 expression in response to hypoxic conditions differed in the young and aging cartilage, respectively, and confirmed that autophagic levels were decreased in the aging compared with the young cartilage. Of note, although decreased autophagy was observed in aging cartilage, the levels of autophagic markers in patients with OA did not differ from those in the aging cartilage. We also detected cell death in the presence of autophagy, and reduced cell death by inhibitors of autophagy in OA chondrocytes. Accordingly, we suggest that during the cartilage pathological process, autophagy may play a cytoprotective and death-promoting role in the pathogenesis of OA.

Materials and methods

Human cartilage sampling. All cartilage samples were collected from human donors who were undergoing surgery at the Department of Orthopaedics, the First Affiliated Hospital of Anhui Medical University, Hefei, China. The specimens were divided into 3 groups: group 1, young cartilage specimens were obtained from 9 patients undergoing lower limb amputation in severe trauma; group 2, 13 aging cartilage specimens, also having no history of joint disease, were harvested from patients with femoral neck fracture who were undergoing femoral head replacement and used as age-matched non-arthritic articular cartilage samples; and group 3, OA cartilage tissues were obtained from 12 patients with early-stage osteoarthritis undergoing total knee arthroplasty. Human tissues were obtained under the approval of the Hospital Ethics Committee of Anhui Medical University. The characteristics of the patients participating in this study are shown in Table I.

Isolation and culture of primary chondrocytes. The cartilage tissue was incubated with trypsin (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 10 min. After the trypsin solution was removed, the tissue slices were treated for 12-16 h with type II collagenase (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco-Life Technologies) with 5% fetal calf serum. The isolated chondrocytes were recovered in DMEM supplemented with 10% fetal calf serum, L-glutamine and antibiotics and were allowed to attach to the culture flasks. The cells were incubated at 37˚C in a humidified gas mixture containing 5% CO_2 balanced with air. The chondrocytes were used in the experiments at confluency (2-3 weeks in primary culture).

Histological and immunohistochemical analysis. The serial sections were stained with hematoxylin and eosin (H&E) in order to observe histological changes in the articular cartilage. Immunohistochemistry (IHC) was performed according to the indirect immunoperoxidase method. In brief, following deparaffinization, hydration and blockage of endogenous peroxidase, the specimens were incubated for 20 min with 10% non-fat milk in phosphate-buffered saline (PBS) in order to block specific sites and then individually incubated at 4˚C overnight with the following primary antibodies: rabbit polyclonal LC3 antibody (NB100-2220, 1:200; Novus Biologicals, LLC, Littleton, CO, USA) and rabbit polyclonal Beclin-1 antibody (ab55878, 1:400; Abcam, Cambridge, MA, USA). After rinsing, the slides were washed, and the sections were incubated with biotinylated goat anti-rabbit secondary

antibody for 30 min at room temperature, and then incubated using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, USA) for 30 min. Finally, the sections were washed and incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate for 2-8 min. Visual impressions of the staining intensity in the cytoplasm and the percentage of immunopositive chondrocytes were recorded by analyzing the digital photomicrographs using ImageJ software.

Transient transfection and confocal microscopy. After the chondrocytes reached 60-70% confluency, they were transfected with green fluorescent protein-light chain 3 (GFP-LC3) plasmid (OriGene Technologies, Inc., USA), using the Lipofectamine 2000 reagent (no. 11668-27; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 24 h after transfection, they were incubated in an O_2 tension of 2% overnight in an Invivo$_2$ Hypoxia Workstation (Ruskinn Technology Ltd., Bridgend, UK) or under normoxic conditions for 24 h, and examined under a confocal microscope. For quantification purposes, the proportion of chondrocytes that contain GFP-LC3 puncta (number of cells with green fluorescent puncta/total number of transfected cells) (15,16), at least 100 green fluorescent chondrocytes should be counted for each sample to obtain statistically significant results.

Western blot analysis. The chondrocytes were isolated from confluent monolayer cultures. Before immunoblotting, protein was quantified using the Bradford method with a BCA detection kit (Thermo Scientific-Pierce, Rockford, IL, USA) and adjusted to equal concentrations across different samples. Equal amounts of protein were separated by SDS-PAGE on precise 10% polyacrylamide gels. Following electrophoresis, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, and then incubated overnight at 4˚C with primary antibodies: rabbit polyclonal LC3 antibody (NB100-2220, 1:2,000; Novus) and rabbit polyclonal Beclin-1 antibody (ab55878, 1:2,000; Abcam). Following incubation, the membranes were washed in TBST buffer for 5 min and probed with anti-rabbit secondary antibody (401393 -2ML, 1:50,000; Millipore) for 1 h at room temperature. They were then washed with TBST 3 times for 5 min. Protein bands of interest were detected using the ECL dectection kit (no. 34094; Thermo Scientific-Pierce). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485, 1:2,000; Abcam) was used as the loading control.

Transmission electron microscopy (TEM). TEM analysis was performed as previously described (17). The chondrocytes were harvested, and fixed in 2.5% glutaraldehyde in phosphate buffer, post-fixed in 2% osmium tetroxide and embedded in Luveak-812 (Nacalai Tesque, Inc., Kyoto, Japan). Ultrathin sections were stained with uranyl acetate for 10 min, then with lead citrate for 10 min, and evaluated under a JEM-1230 electron microscope (Jeol Ltd., Tokyo, Japan).

Monodansylcadaverine (MDC) staining. Three groups of chondrocytes were seeded on coverslips overnight and then

Table I. Characteristics of the patients participating in this study.

Characteristics	OA cartilage (n=12)	Aged cartilage (n=13)	Young cartilage (n=9)
Age (mean ± SD)	65.4±8.6 years	63.4±6.2 years	22.4 ±6.2 years
Gender (female/male)	7/5	6/7	4/5
Diagnosis	Osteoarthritis	Femoral neck fracture	Destructive limb injury
Medications	Total knee arthroplasty	Total hip replacement	Amputation
OA grade	II-III	I-II	I
Mankin score	4-7	0-2	0

OA, osteoarthritis; SD, standard deviation.

treated with rapamycin or 3-methyladenine (3-MA) (both from Sigma-Aldrich, Shanghai, China). After autophagy activation or inhibition, the cells were incubated with 0.05 mM MDC (Sigma-Aldrich, Shanghai, China) in PBS at 37˚C for 10 min, then washed 3 times with PBS and fixed with a solution of 4% paraformaldehyde for 30 min. The coverslips were examined under a fluorescence microscope (XSZ-D2; Olympus, Tokyo, Japan). To quantify autophagic cells after treatment, we counted the number of autophagic cells demonstrating MDC-labeled particles among 200 cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells ($2x10^4$) were plated into a 96-well plate and cultured for >12 h. The chondrocytes were then subjected to autophagy activation or inhibition using pharmacological tools for 24 h at various concentrations, and then incubated with MTT (0.5 g/l) for 4 h. The formazan precipitate was dissolved in 200 μl dimethyl sulfoxide and the absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Acridine orange (AO) staining and quantification by flow cytometry. AO is a widely used to visualize acidic vesicular organelles (AVOs) (18). AO is a fluorescent dye that stains DNA and the cytoplasm bright green. At low pH, AO emits red fluorescence with intensity proportional to the degree of acidity. To detect the cell death rate, following the pharmacological activation or inhibition of autophagy, the cells were washed twice with PBS, fixed with 4% paraformaldehyde and stained with AO (Molecular Probes, Eugene, OR, USA) at 1 μg/ml for 15 min. They were then washed with PBS to remove the unbound dye. AVO formation in the AO-stained cells was measured by flow cytometry, and the cells were analyzed on a flow cytometer using CellQuest software.

Quantification of DNA hypoploidy and cell cycle phase analysis by flow cytometry. When the chondrocytes reached 60-70% confluency, we treated the cells with 3-MA or rapamycin (Sigma-Aldrich, Shanghai, China). Following treatment for the desired amount of time, the cells were harvested. The digested cells were then washed 3 times in PBS, fixed with ice cold 70% ethanol and then incubated at -20˚C overnight. Subsequently, the fixed cells were centrifuged and washed 3 times with PBS. The cells were resuspended in 250 μl prop-

idium iodide (50 mg/ml) and incubated in the dark at room temperature for 3 h. The cells were then analyzed on a flow cytometer using CellQuest software.

Statistical analyses. All data and results presented in this study were derived from at least 3 independent experiments. The data are expressed as the means ± SD. The significance of the data was evaluated by one-way ANOVA. Values of P<0.05 were considered to indicate statistically significant differences.

Results

Differential expression of Beclin-1 and LC3 in human articular cartilage tissues. To determine the role of autophagy in the development of OA, we examined Beclin-1 and LC3 expression in young, aging and OA cartilage by IHC in the upper zone of the cartilage (including the superficial and middle zones). IHC analysis indicated that Beclin-1 and LC3 were ubiquitously expressed in the young cartilage group, but lower expression levels were observed in the aging cartilage. Cartilage samples were evaluated by staining with H&E (Fig. 1A-a-c) and Beclin-1 and LC3 expression levels were determine by IHC (Fig. 1A-d-g). The percentage of chondrocytes positive for Beclin-1 was substantially decreased in the aging group (21.5±6.1%) compared with the young group (64.8±4.4%), while the percentage of LC3-positive cells in the young and aging groups (57.2±5.6 and 24.3±5.7%, respectively) indicated a statistically significant decrease in aging cartilage (P<0.05) (Fig. 1B). These results indirectly suggest a decline in autophagic activity in aging tissue. Of note, we found that the expression of autophagic proteins was not reduced in patients with OA. In the OA group, the percentages of Beclin-1- and LC3-positive cells were 44.1±5.6 and 62.4±7.6%, respectively (Fig. 1B).

Differences in the autophagic response of cultured articular chondrocytes. We used human primary chondrocytes transiently expressing GFP-LC3 to quantify autophagosome formation. The results revealed that the percentage of chondrocytes with GFP-LC3 puncta structures was low (Fig. 2A). When comparing aging and OA chondrocytes, the percentage of chondrocytes with GFP-LC3 puncta structures did not differ. The results of electron microscopy also revealed few autophagosomes in these 3 groups of chondrocytes. Western
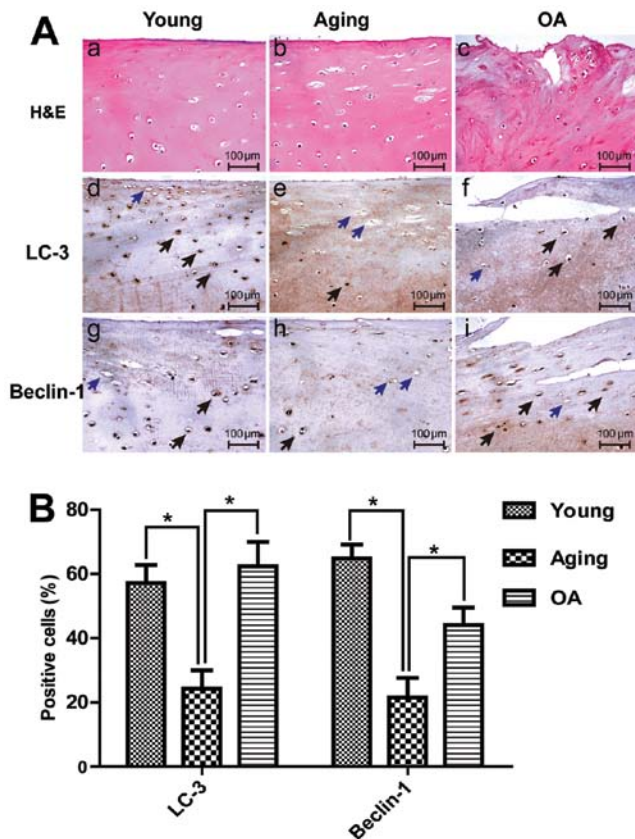
Figure 1. Expression of Beclin-1 and light chain 3 (LC3) in human articular cartilage tissue. (A) (a-c) Hematoxylin and eosin (H&E)-stained sections representative of cartilage samples from young, aging and osteoarthritis (OA) donors. (d-f) Immunohistochemical analysis with LC3, (g-i) immunohistochemical analysis with Beclin-1; immunopositive chondrocytes are indicated by black arrows and immunonegative ones by blue arrows. (B) Quantification of Beclin-1- and LC3-positive cells in cartilage. The percentage of immunopositive chondrocytes was recorded by analyzing the digital photomicrographs on a computer loaded with ImageJ software. The results represent the means ± SD of young and OA specimens. *P<0.05 vs. aging cartilage.
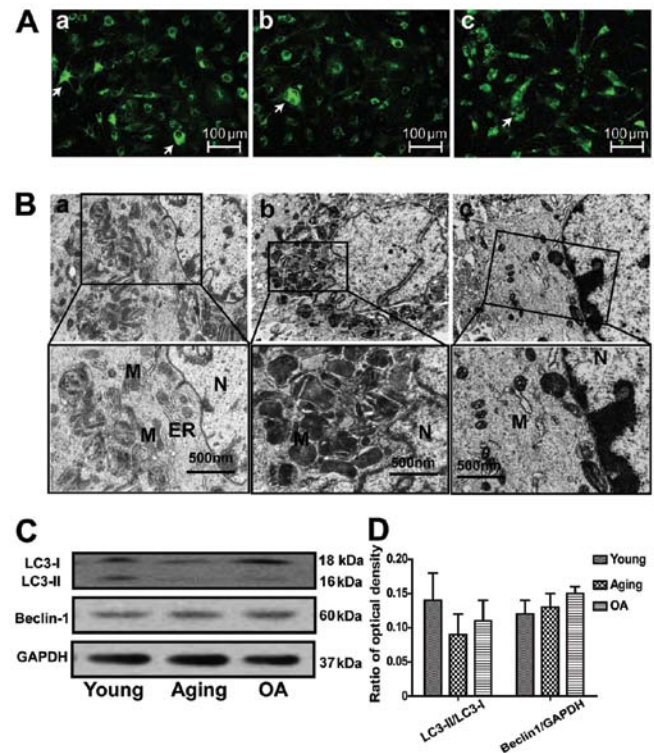

Figure 2. Levels of autophagic activity in chondrocytes cultured under normoxic conditions. (A) Chondrocytes with green fluorescent protein-light chain 3 (GFP-LC3) puncta structures in (a) the young group; (b) the aging group; (c) the osteoarthritis (OA) group; White arrows represent cells with GFP-LC3 puncta structures. No differences were observed between the 3 groups of chondrocytes as regards GFP-LC3 puncta structures. (B) Electron microscopy investigations showed a marked difference between the chondrocytes in the 3 groups. (a) In the young group, a large nucleus (N) surrounded by a well-defined cytoplasm and normal endoplasmic reticulum (ER), mitochondria (M) and abundant perinuclear filaments were observed; (b) in the aging group, an elongated shape nucleus, prominent rough endoplasmic reticulum, Golgi complex and a lower number of mitochondria were observed; and (c) the OA chondrocytes contained an irregular-shaped nucleus, a swollen rough endoplasmic reticulum, no Golgi complex and a small number of mitochondria. (C and D) We evaluated LC3 and Beclin-1 protein expression by western blot analysis in the young, aging and OA chondrocytes from 4 donors. One representative western blot is shown. The optical density of densitometric analysis showed that the optical density of LC3-II/LC3-I and Beclin-1/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was not significantly altered in the chondrocytes from young, aging and OA donors (P<0.05).

blot analysis also revealed no significant differences in the expression levels of autophagic proteins in the chondrocytes (Fig. 2C and D). Taken together, these results indicate that autophagic activity was low and did not differ between the 3 groups of cultured chondrocytes *in vitro*.

Hypoxia plays an essential role in autophagy in chondrocytes. Chondrocytes grow in a hypoxic environment *in vivo* (19). Thus, in this study, we further examined the role of hypoxia in autophagy in cultured chondrocytes. In the young and OA chondrocytes, the numbers of GFP-LC3 puncta structures were markedly increased, but in the aging group, no significant change was observed (Fig. 3A). Compared with the groups exposed to normoxic conditions, the percentage of cells with GFP-LC3 puncta structures in the groups exposed to hypoxic conditions was significantly increased. We subsequently used electron microscopy to observe the changes in the ultrastructure of chondrocytes following hypoxia-induced autophagy. A large number of autophagosomes with degraded organelles was observed in the young chondrocytes (Fig. 3C-a). By contrast, the aging chondrocytes contained few autophagosomes. Of note,

in the OA chondrocytes, we found characteristic structures of autophagic cell death (Fig. 3C-b); these cells with autophagic vacuoles contained cell fragments, autolysosomes and few or no chromatin was condensed. In addition, our results revealed a significant increase in the expression of LC3-II protein in the young and OA groups; however, no significant changes were observed in the aging group (Fig. 3D and E).

Effect of rapamycin and 3-MA on autophagic of chondrocytes. In this study, we demonstrated that autophagic activity was increased under hypoxic conditions compared with normoxic conditions. Thus, we induced autophagy under normoxic conditions and inhibited autophagy under hypoxic conditions. As illustrated in Fig. 4A-d-f, following the treatment of the chondrocytes with 10 µM rapamycin, which has been
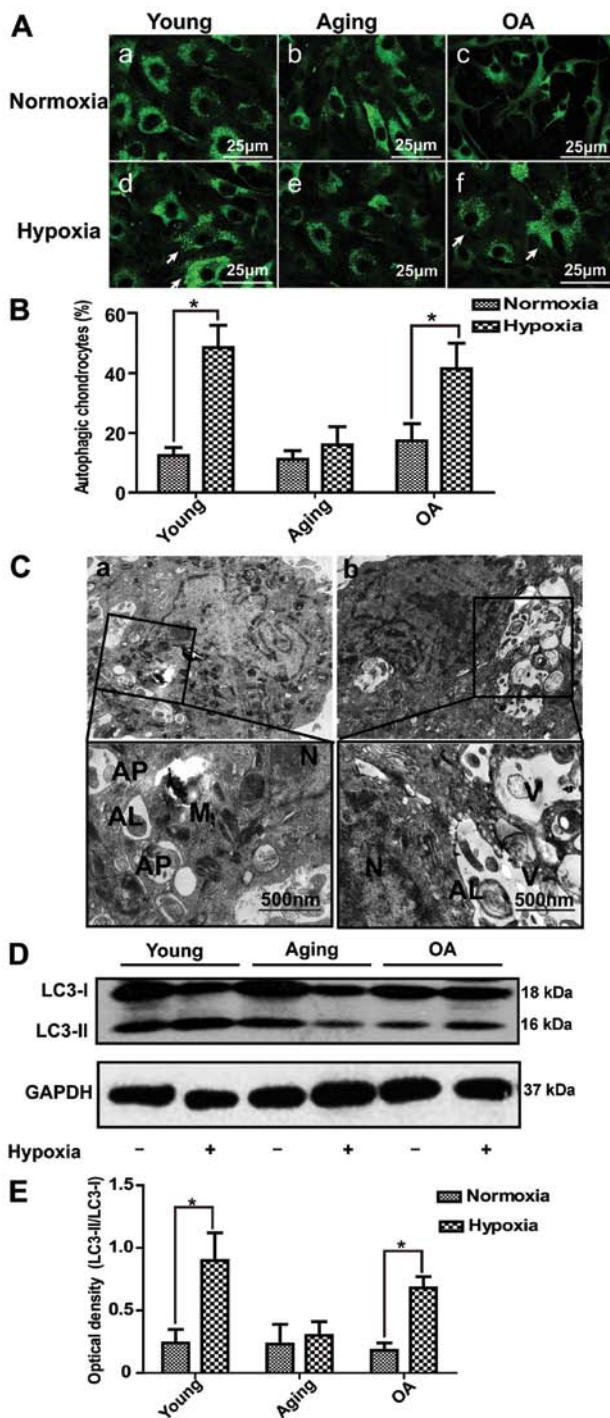
Figure 3. Hypoxia plays an essential role in autophagy in chondrocytes. (A and B) Analysis of green fluorescent protein-light chain 3 (GFP-LC3) puncta structures by confocal microscopy. Percentage of cells with GFP-LC3 puncta structures was as follows: young group: normoxia/hypoxia, 12.4±2.6/48.5±7.5%; aging group: normoxia /hypoxia, 11.1±2.9/15.9±6.1%; osteoarthritis (OA) group: normoxia/hypoxia, 17.3±5.7/41.4±8.6%. The numbers of puncta structures were dramatically increased in the young and OA chondrocytes. The results are the means ± SD of young and OA chondrocytes. *P<0.01 vs. normoxia. (C) (a) Electron microscopy demonstrating autophagic chondrocytes, a large number of autophagosomes (AP) with degraded organelles from the young chondrocyte group. (b) Autophagic chondrocyte death in the OA group; the cells including autophagic vacuoles (V) contained cell fragments, autolysosomes (AL) and few or no chromatin was condensed. M, mitochondria. (D and E) LC3 protein expression was determined by western blot analysis in young, aging and OA chondrocytes. The optical density of densitometric analysis showed a 3.75-fold increase in the young group and a 3.78-fold increase in the OA group under hypoxic conditions compared with normoxic conditions. In addition, the optical density of LC3-II/LC3-I was not significantly altered in the aging chondrocytes from the donors (*P<0.05).
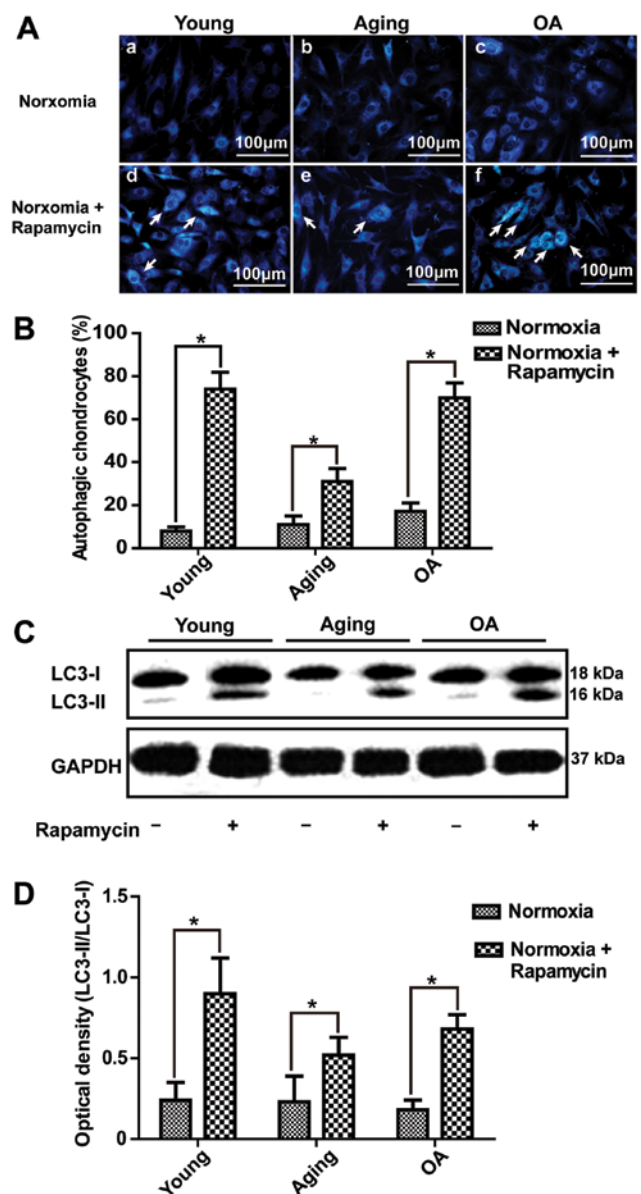
Figure 4. Effect of rapamycin on autophagy of chondrocytes. (A and B) Mono-dansylcadaverine (MDC)-labeled vesicles (indicated by arrows) were induced by rapamycin. MDC was concentrated in the spherical structures distributed in the cytoplasm. The chondrocytes have weak and diffuse MDC staining in untreated cells. To quantify autophagic cells after treatment, we counted the number of autophagic cells demonstrating MDC-labeled particles among 200 cells. Values are the means ± SD from at least 3 independent experiments. *P<0.05 vs. normoxia. (C and D) We evaluated the effect of rapamycin on light chain 3 (LC3)-II/LC3-I expression by western blot analysis. Densitometry analysis revealed a significant increase in LC3-II/LC3-I expression by 3.75-, 2.26- and 3.78-fold in the young, aging and osteoarthritis (OA), respectively in the rapamycin-treated chondrocytes under hypoxic conditions compared with normoxic conditions. Values are the means ± SD (*P<0.05).

reported to be a potent inducer of autophagy (13,20,21), under normoxic conditions, the number of vesicles was increased by MDC staining (Fig. 4B). Western blot analysis also indicated that the expression of LC3-II was significantly increased in the young and OA chondrocytes (Fig. 4C). This result indicated that autophagy in young and OA chondrocytes was more easily induced than in aging chondrocytes (Fig. 4D). Under hypoxic conditions, following the pre-treatment of the chondrocytes
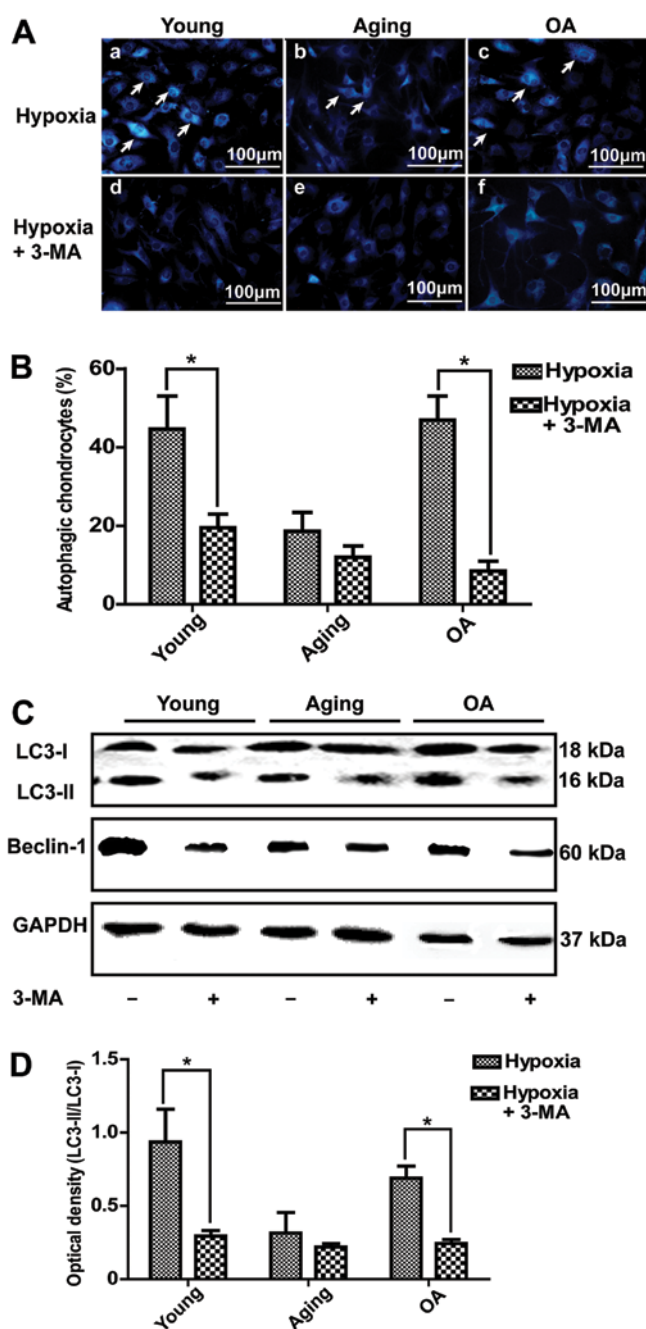
Figure 5. Effect of 3-methyladenine (3-MA) on autophagy of chondrocytes. (A and B) Monodansylcadaverine (MDC) staining was used to quantify autophagic chondrocytes, arrows represent autophagic cells. The quantification results of MDC incorporation into the cells indicated that 3-MA decreased autophagy from 46.9±6.1 to 8.5±2.5% in osteoarthritis (OA) group, compared with the young and aging group (from 44.6±8.4 to 19.5±3.5%, from 18.6±4.8 to 12.0±2.9%, respectively). (C and D) We evaluated the effect of 3-MA on light chain 3 (LC3)-II/LC3-I expression by western blot analysis. Densitometry analysis revealed a significant decrease in LC3-II/LC3-I expression by 3.17-, 1.55- and 5.52-fold in the young, aging and OA groups, respectively under normoxic conditions compared with hypoxic conditions. Values are the means ± SD (*P<0.05).

with 3-MA, which is one of the most commonly used inhibitors of autophagy (22,23), the number of autophagic vacuoles stained by MDC was much lower (Fig. 5A and B). Moreover, LC3-II and Beclin-1 expression significantly decreased in the young and OA chondrocytes (Fig. 5C and D).
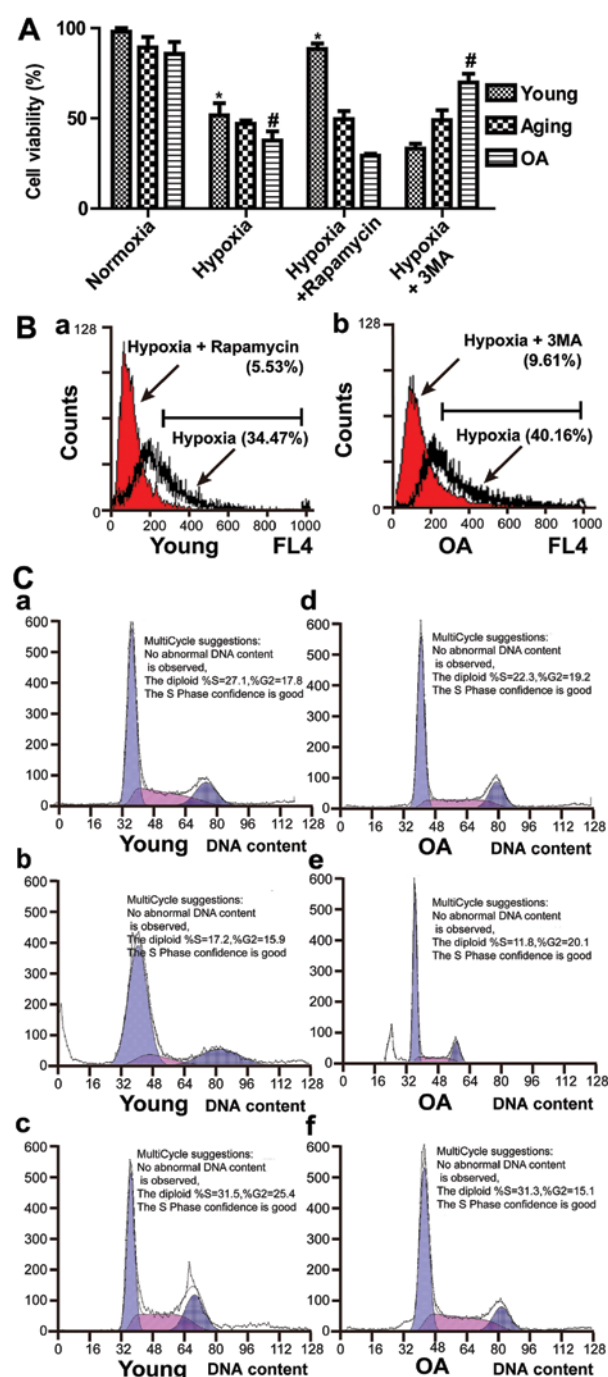

Figure 6. Effect of autophagy on cell viability and cell cycle of chondrocytes. (A) Effect of rapamycin or 3-methyladenine (3-MA) on the viability of chondrocytes. Cell viability was determined by MTT assay. The cell survival rate changed from 51.57±6.92 to 88.27±3.15% in the young chondrocytes after the induction of autophagy, and the cell survival rate changed from 37.69±5.17 to 69.86±4.81% in osteoarthritis (OA) chondrocytes after the inhibition of autophagy (*P<0.05). (B) Flow cytometry was performed to detect cell death by acridine orange (AO) staining. (a) The cell death rate changed from 34.47 to 5.53% after the induction of autophagy in young chondrocytes. (b) The cell death rate changed from 40.16 to 9.61% after the inhibition of autophagy in OA chondrocytes. (C) Quantification of DNA hypoploidy and cell cycle phase analysis by flow cytometry. (a) Young chondrocytes under normoxic conditions: the majority of cells were in the G0/G1 phase. (b) Young chondrocytes under hypoxic conditions: the number of cells decreased in S phase. (c) Young chondrocytes following treatment with rapamycin under hypoxic conditions: the number of cells decreased in the S and G2 phase. (d) OA chondrocytes under normoxic conditions: many of the cells were in the G0/G1 phase. (e) OA chondrocytes under hypoxic conditions: the number of cells decreased in the S phase. (f) OA chondrocytes following treatment with 3-MA under hypoxic conditions: the number of cells was decreased in the S phase, and the number of cells in the sub-G1 phase declined.

Effects of autophagy on cell viability and cell cycle of chondrocytes. To extend our analysis, we intervened in the autophagic pathway to examine the effects on cell viability. In particular, we examined whether autophagy contributes to the cell survival or cell death process. Cell viability was determined by MTT assay. The cell survival rate increased significantly in the young chondrocytes after the induction of autophagy (P<0.05). In addition, the cell survival rate changed from 37.69±5.17 to 69.86±4.81% in the OA chondrocytes after the inhibition of autophagy (Fig. 6A). Flow cytometry was performed to detect cell death by AO staining. As shown in Fig. 6B-a, the cell death rate changed from 34.47 to 5.53% after the induction of autophagy in young chondrocytes. In the OA chondrocytes, by contrast, it changed from 40.16 to 9.61% after the inhibition of autophagy (Fig. 6B-b). These results demonstrated that autophagy was beneficial to cell survival in young chondrocytes but led to cell death in OA chondrocytes. To further clarify the possible mechanisms by which autophagy affected the viability of chondrocytes, we used flow cytometry for the quantification of DNA hypoploidy, analysis of the cell cycle and the measurement of cell sub-G1 peaks. In the young chondrocytes, the majority of cells were in the G0/G1 phase of the cell cycle (Fig. 6C-a). Flow cytometry demonstrated that rapamycin prevented the accumulation of subdiploid cells (Fig. 6C-b and c). These findings suggest that increased autophagosome formation is required for chondrocyte survival in young cartilage. As shown in Fig. 6C-e, autophagy was critical for the OA chondrocyte cycle progression from the G2/M to the G1 phase under hypoxic conditions. The sub-G1 population of the cell cycle is represented as total cell death, and we found that the sub-G1 population in the OA chondrocytes decreased from 16.24% to 4.47% following treatment with 3-MA under hypoxic conditions (Fig. 6C-e and f).

Discussion

The aim of this study was to confirm that autophagy plays a dual role in articular cartilage tissue. Firstly, we confirmed that autophagic activity was downregulated in the chondrocytes of aged articular cartilage. Surprisingly, we found that the expression of autophagic-related proteins was not decreased in the tissues of patients with OA. In addition, we also found that hypoxia may induce the autophagy of chondrocytes. Another observation was that rapamycin may activate autophagy, which plays a protective role in young chondrocytes. However, the excessive activation of autophagy led to autophagic cell death in OA chondrocytes *in vitro*.

The present study demonstrated that autophagic activity declined in the aging articular cartilage. We found that LC3-II conversion occurred more easily in young chondrocytes than in aging chondrocytes. In early autophagy, LC3-I is converted to LC3-II through lipidation by the ubiquitin-like system, which correlates with the extent of autophagosome formation (24,25). Therefore, decreased autophagic activity may participate in the pathological process of cartilage aging. Aging is one of the main factors in the pathogenesis of OA, and older patients who experience a joint injury develop OA much more rapidly than young patients with a similar knee injury (26). During aging, the efficiency of autophagic degradation is declined and intracellular waste products are accumulated. Furthermore, there is a general consensus that the function of autophagy is decreased during aging (27-29). This observation is consistent with the notion that basal autophagic activity is decreased with age, thus contributing to the accumulation of damaged macromolecules and susceptibility to aging-related diseases (30).

To our surprise, the autophagic markers were found to be increased in patients with OA. Beclin-1 and LC3 expression levels in OA cartilage were significantly increased in the upper zone compared with those in aging articular cartilage. These results are not completely consistent with those of Caramés *et al* (12). They reported that the expression of ULK1, Beclin-1 and LC3 in OA cartilage was significantly decreased in the superficial zone. We speculated that their results may be due to the wear and tear with the loss of chondrocytes in the superficial zone. However, we found the cell clusters which localized in the middle zone in OA cartilage showed a strong expression of Beclin-1 and LC3. In addition, the results of GFP-LC3 transfection indicated that the chondrocytes in OA were more easily susceptible to autophagy than those in the aging group, which further demonstrated that autophagic activity in OA was higher than the aging group.

We found that autophagy may play a protective role in young cartilage. In our experiments, rapamycin-induced autophagy protected chondrocytes in the young group from death under hypoxic conditions, to a certain extent. Autophagy is a mechanism for the turnover of proteins and elimination of damaged organelles to maintain cell homeostasis. The induction of autophagy under pathological conditions is generally considered to play a cytoprotective role in young cartilage. Recent studies provide compelling evidence that autophagy protects against diverse pathologies, such as neurodegenerative diseases (31), cancer (32), aging (33) and heart diseases (34). Therefore, we suggested that in young articular cartilage, autophagy was activated as an adaptive response to hypoxic conditions. These results indicate that compromised autophagy represents a novel mechanism in the development of cartilage degeneration.

The most intriguing finding of our study was that autophagic chondrocyte death occured in OA. Roach *et al* (11) described a morphological change of cell death in articular cartilage which was similar to autophagic cell death. The present study first reported that this type of death is classic autophagy in chondrocytes. We detected cell death in the presence of autophagy, and demonstrated the reduction in cell death by the inhibition of autophagy. Such findings are consistent with most basic characteristics of autophagic cell death (35). Autophagic cell death is morphologically defined as a type of cell death. This autophagy-dependent non-apoptotic cell death is defined as autophagic cell death, or type II programmed cell death (PCD) (36). Therefore, our results provided a starting point to study autophagic chondrocyte death in OA.

In addition, although our results demonstrated that autophagy induced chondrocyte survival and death in different stages of OA progression. Certain studies have found that autophagy can promote cell survival or cell death, depending on the type of cellular stress (35). Autophagy has also been reported to play a role in cell survival under other types of stress, such as exposure to DNA-damaging reagents (37), endoplasmic reticulum stress (38) and radiation (39). Exposure to treatment

conditions, such as hypoxia (40) and arsenic trioxide (41) have been reported to induce autophagic cell death.

In conclusion, the present study demonstrates that rapamycin-induced autophagy does not lead to chondrocyte death in young patients, while it can induce cell death by autophagy in the patients with OA. We described a novel and paradoxical role for autophagic cellular degradative pathways in OA cartilage. These results suggest that autophagy may play both a cytoprotective and death-promoting role in chondrocytes. Therefore, the dual role of autophagy in cytoprotection and cell death, as well as its impact on longevity is one of the most fascinating features of this process, which clearly has a direct impact on the age-related development of cartilage degeneration. Understanding the role of autophagy in chondrocyte responses should provide new targets for attenuating and preventing the development of OA.

Acknowledgements

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