Endoplasmic Reticulum Stress-induced Apoptosis Contributes To Articular Cartilage Degeneration Via C/EBP Homologous Protein

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Introduction: Loss of homeostasis due to chondrocyte apoptosis leads to the destruction of articular cartilage characteristic of osteoarthritis (OA). Apoptosis is triggered when death stimuli activate one of three distinct cell death pathways; the plasma membrane death receptor-, the mitochondrial-, or the endoplasmic reticulum (ER) stress pathway. When ER stress, i.e., the excessive accumulation of unfolded proteins in ER, endangers homeostasis, apoptosis is induced by C/EBP homologous protein (Chop). In OA cartilage, Chop expression and apoptosis increase as degeneration progresses. In cultured chondrocytes, the ER stress inducer tunicamycin (TM) mediates Chop expression and chondrocyte apoptosis, decreases aggrecan (Acan) and collagen type II (Col2) mRNA expression. Our hypothesis was that Chop may play a direct role in ER stress-induced apoptosis that resulted in cartilage degeneration. We studied this hypothesis in Chop-knockout (Chop-/-) and wild-type (Chop+/+) mice murine model of OA and cultured chondrocytes.

Methods: We induced experimental OA in Chop+/+ and Chop-/- mice by medial collateral ligament transection and meniscectomy. The whole knee joint was harvested at 0, 4, 8, and 12 weeks after surgery (n=10 at each time point). The histological severity of cartilage degeneration was evaluated by the modified Mankin score with Safranin-O fast-green staining. Chondrocyte apoptosis and ER stress in cartilage were evaluated semiquantitatively by the percentage of cells positive for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and immunohistochemical staining of Chop, the spliced form of X-box binding protein 1 (Xbp1s) and 78 kDa glucose-regulated protein (Grp78), respectively. The anabolic function of chondrocytes was analyzed by immunohistochemistry for Col2 and measured semiquantitatively with a staining H-score. The score was multiplied by the median percentage staining area score for each category (none, weak, moderate, and strong) in order to derive an overall staining score. In our in vitro experiments, articular primary chondrocytes from Chop+/+ and Chop-/- mice were dissociated enzymatically and incubated for 12 hours with TM. Using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis, the levels of ER stress were evaluated by the expression of Chop, Grp78 and Xbp1s. Apoptosis of cultured chondrocytes was analyzed with TUNEL staining and enzyme-linked immunosorbent assay (ELISA). The expression of Col2 and Acan mRNA was studied by quantitative RT-PCR analysis. All data are expressed as the mean ± 95% confidence intervals. The Student's t-test was used to analyze differences between Chop+/+ and Chop-/- mice. It was also used to analyze the differences between Chop+/+ and Chop-/- chondrocytes, and between TM- and TM+. One-way analysis of variance (ANOVA) with Bonferroni post hoc correction was performed to analyze the differences among four time points. Differences of P<0.05 were considered statistically significant.

Results: Cartilage degeneration was observed during the postoperative observation period in both Chop+/+ and Chop-/- mice. The modified Mankin scores were significantly lower in Chop-/- than
Chop+/+ mice at 4, 8, and 12 weeks (P<0.05, Fig.1). TUNEL-positive cells appeared gradually during the 12-week observation period in both mice. The percentage of TUNEL-positive cells was significantly lower in Chop-/- mice than Chop+/+ mice at weeks 4, 8, and 12 (P<0.05, Fig.2). The percentage of Chop-positive cells at week 0, 4, 8, and 12 was 3.1%, 12.8%, 15.9%, and 27.9% in Chop+/+ mice; no Chop-positive cells were observed in Chop-/- mice. The differences between the two groups were significant (P<0.05). The percentage of Xbp1s-positive cells at weeks 0, 4, 8, and 12, was 16.3%, 38.8%, 33.2%, and 31.2% in Chop+/+ and 14.3%, 35.0%, 34.9%, and 32.5% in Chop-/- mice. Their percentage was significantly higher in both groups at weeks 4, 8 and 12 than at week 0 (P<0.05), and there was no significant difference between the two groups. The percentage of Grp78-positive cells at week 0, 4, 8, and 12 was 24.7%, 42.8%, 44.2%, and 41.0% in Chop+/+ and 26.8%, 45.0%, 46.9%, and 43.1% in Chop-/- mice. Their percentage at 4, 8, and 12 was significantly higher than at week 0 (P<0.05); the difference between the two groups was not significant. The staining H-score at weeks 0, 4, 8, and 12 was 249.0, 187.6, 151.2, and 116.2 in Chop+/+ mice and 256.5, 225.2, 195.2, and 163.6 in Chop-/- mice. These scores were significantly higher in Chop-/- than Chop+/+ mice (P<0.05). In vitro, the expression of Chop mRNA was 20.5 times higher in TM-treated than untreated Chop+/+ chondrocytes. Neither Chop mRNA nor Chop protein expression was found in Chop-/- chondrocytes. The expression of Grp78 mRNA was 13.5 times higher in TM-treated Chop+/+ and 12.6 times higher in TM-treated Chop-/- chondrocytes than in TM-untreated cells, and the protein expression was increased equally in Chop+/+ and Chop-/- chondrocytes. The expression of Xbp1s mRNA was similarly up-regulated by TM in Chop+/+ and Chop-/- chondrocytes. The number of TUNEL-positive cells with TM treatment was increased in Chop+/+ and Chop-/- chondrocytes compared to TM-untreated chondrocytes, but it was less in Chop-/- than Chop+/+ chondrocytes (Fig.3). ELISA showed that TM significantly increased the level of apoptosis in Chop+/+ and Chop-/- cells, but it was significantly lower than in Chop-/- than Chop+/+ chondrocytes (P<0.05). After TM treatment, Col2a1 mRNA expression was reduced by 71.2% in Chop+/+ and by 50.0% in Chop-/- chondrocytes compared to TM-untreated chondrocytes, and the expression was significantly higher in Chop-/- than Chop-/+ chondrocytes (P<0.05). TM reduced the expression of Acan mRNA by 60.4% in Chop+/+ and by 37.1% in Chop-/- chondrocytes compared to TM-untreated chondrocytes, and the expression was significantly higher in Chop-/- than Chop+/+ chondrocytes (P<0.05).

**Discussion:** In conclusion, our results indicate that Chop plays a direct role in chondrocyte apoptosis and that Chop-mediated apoptosis contributes to the progression of cartilage degeneration in mice. The most important limitation of the present study is that we cannot rule out the existence of ER stress-induced pathways other than Chop. The ER stress-induced apoptosis was also induced by c-Jun N-terminal kinases. In addition, apoptosis had been induced by the crosstalk of ER stress and oxidative stress. We need to investigate the relationship between these mediators and ER stress in our OA model.

**Significance:** In clinical practice, the treatment of OA is challenging and in many cases without success. Our findings suggest that Chop may become a molecular therapeutic target for OA.
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**Figure 1**

Time course of cartilage degeneration in a murine model of OA. Cbfa1+/−Ctsc−/− mice showed no cartilage degeneration. Histologically, cartilage degeneration began at 4 weeks and progressed significantly during the 12-week observation period in Chacp+/− mice. The cartilage degeneration was more severe in Chacp−/− mice. The modified Harris scores were significantly lower in Chacp+/− mice at 4, 8, and 12 weeks. Data are expressed as the mean (SD), ± 95% confidence intervals (error bars). Scale bar = 100 μm, original magnification x200. a: P < 0.001 vs control, b: P < 0.001 vs week 0, c: P < 0.001 vs week 6 in each group of mice by ANOVA with Bonferroni post-hoc correction. P = 0.001 vs Chacp−/− mice by the Student's t-test.

**Figure 2**

Time course of TUNEL-positive cells in a murine model of OA. The degree of apoptosis was evaluated by TUNEL staining and was analyzed quantitatively by the ratio of TUNEL-positive cells/1000 mean nucleotide group and time point. (A) TUNEL-positive cells in cartilage samples gradually increased during the 12-week observation period in Chacp+/− and Chacp−/− mice. (B) The number of TUNEL-positive cells was significantly lower in Chacp+/− mice at weeks 4, 8, and 12 weeks. Data are expressed as the mean (SD), ± 95% confidence intervals (error bars). Scale bar = 100 μm, original magnification x200. a: P < 0.001 vs control, b: P < 0.001 vs week 0, c: P < 0.001 vs week 6 in each group of mice by ANOVA with Bonferroni post-hoc correction. P = 0.001 vs Chacp−/− mice by the Student's t-test.

**Figure 3**

Evaluation of apoptosis in cultured chondrocytes. Three independent experiments were performed, and the data was analyzed in triplicate. (A) TUNEL-positive cells were increased by TNF in Chacp+/− and Chacp−/− chondrocytes. The increase was less in Chacp−/− than Chacp+/− cells. 20 μg/mL IGF-I showed that the level of apoptosis increased by TNF in both groups. This increase was less in Chacp−/− than Chacp+/− cells. Scale bar = 100 μm, original magnification x200. Data are expressed as the mean (SD), ± 95% confidence intervals (error bars). a: P < 0.001 vs control, b: P < 0.001 vs Chacp+/− (Student's t-test).