Oxidative Stress-induced Apoptosis And Matrix Loss Of Chondrocytes Is Inhibited By Eicosapentaenoic Acid.

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Introduction: Osteoarthritis (OA) is the most common chronic joint disease in the elderly population. OA is characterized by the progressive destruction and loss of the matrix of articular cartilage due to the unbalanced anabolic and catabolic activities of chondrocytes. Apoptosis of chondrocytes is involved in the pathological condition of OA, and has been reported to be correlated to the severity of OA and associated with disease progression. Degeneration of chondrocytes has been reported to be correlated with the loss of articular cartilage caused by increased degradation of the matrix. Eicosapentaenoic acid (EPA) is an antioxidant. It is an n-3 polyunsaturated fatty acid present in fish oil. EPA is used widely for cardiovascular problems or the alleviation of Alzheimer’s disease. N-3 fatty acids have been shown to suppress the expression of MMP and inflammatory cytokines in vitro (1,2,3,4). However, how EPA prevents cartilage degradation in OA progression is not known, a lacuna that we aimed to study in vitro and in vivo.

Methods: Cell culture: Normal human chondrocytes-knee (NHAC-kn) cells were cultured at 37°C and 5% CO2 in Dulbecco’s modified Eagles’s medium (DMEM). EPA was added to NHAC-kn cells and was preincubated for 8 h. Sodium nitro prusside (SNP) was added to culture media and incubated for a further 30 min or 12 h.

Western blotting: Chondrocytes were lysed, supplemented with a protease and phosphatase inhibitor mix. Each sample was electrically transblotted onto a membrane. Anti-human phospho-p53 at ser-46, poly (ADP-ribose) polymerase (PARP), and cleaved caspase 3 were used as primary antibodies. Signals were visualized in Chemilumino Analyzer.

Real-time polymerase chain reaction (PCR) analyses: Chondrocytes were cultured in six-well plates with EPA and SNP. RNA was extracted and reverse transcribed to first-strand complementary DNA (cDNA). Real-time PCR was performed and carried out using the 2ΔΔCt method. The specific primer of MMP13 were used.

Analyses of DNA fragmentation: DNA fragmentation was evaluated using an APO-DIRECT kit according to manufacturer instructions and was analyzed by flow cytometry.

Animals: C57BL/6J male mice were used in this experiment. Destabilization of the medial meniscus (DMM) surgery was carried out on right knee joints. Corn oil or corn oil with EPA was injected into the knee joint every week for 12 weeks.

TUNEL staining: Apoptosis was evaluated using an In situ Apoptosis Detection kit according to manufacturer instructions.

Immunohistochemical analyses: Deparaffinized sections were digested with proteinase and treated with hydrogen peroxide. Sections were treated with anti-MMP13 antibodies. The signal was developed as a
brown reaction product using the peroxidase substrate 3,3′-diaminobenzidine, and sections examined under light microscopy.

**Results:** Flow cytometry demonstrated that percent apoptosis increased to 43.0% upon SNP treatment, and that EPA reduced percent apoptosis to 22.9% (Figure 1A). Western blotting demonstrated that they were increased by SNP treatment. However, exogenous EPA treatment decreased all of them (Figure 1B, 1C). Real-time PCR demonstrated that expression of MMP13 was increased significantly upon SNP treatment. However, MMP13 expression was decreased significantly upon treatment with SNP and EPA (Figure 1D).

Staining with Safranin O and Fast Green showed that matrix degradation did not occur in the sham-operated (Figure 2A). DMM surgery mice and mice injected with corn oil showed surface degradation of cartilage tissue and depression of matrix staining within the upper-third of cartilage (Figure 2B, C). However, mice injected with EPA did not show matrix degradation or depression of matrix staining (Figure 2D).

TUNEL staining demonstrated that TUNEL-positive cells were ubiquitously expressed in the DMM cartilage injected with corn oil (control), but not in cartilage injected with corn oil and EPA (EPA) (Figure 3A, B). The percentage of TUNEL-positive apoptotic cells decreased significantly in cartilage injected with EPA (Figure 3C). Immunohistological analyses demonstrated that MMP13 was ubiquitously expressed in the DMM cartilage injected with corn oil (control), but not in cartilage injected with corn oil and EPA (EPA) (Figures 3D, E). The average percentage of MMP13-positive cells decreased significantly in cartilage injected with EPA (Figure 3F).

**Discussion:** Nitric oxide (NO) is a free radical that undergoes various reactions in tissues. Overproduction of NO has been reported to induce the apoptosis of chondrocytes. Therefore, we analyzed the EPA functions in response to NO-induced oxidative stress in the present study. NO induces the phosphorylation of p38MAPK, which leads to p53 phosphorylation. p53 phosphorylation induces cleavage of caspase-3 and PARP, leading to apoptosis (5,6). Our results demonstrated that EPA reduced the apoptosis of chondrocytes by inhibition of the phosphorylation of p38MAPK and p53. These results suggest that EPA protects chondrocytes from apoptosis induced by oxidative stress through the p38MAPK-p53 pathway.

Curtis et al. reported that n-3 fatty acids reduce the expression of MMP-3 and MMP-13 in human OA cartilage explant cultures (1). In the present study, expression of MMP3 and MMP13 was increased by oxidative stress, and exogenous EPA treatment significantly reduced the expression of MMPs. These results are in accordance with those of previous studies.

Rodeny et al. and Knot et al. reported that n-3 fatty acids prevents OA progression in vivo (3,4). However, these studies did not show evidence of how n-3 fatty acids reduce OA progression. Therefore, we investigated how EPA prevents OA progression in vivo. We showed that intra-articular injection of EPA protects chondrocytes from apoptosis and reduces the expression of MMP13 in a mouse model of OA. These findings indicate how EPA protects against OA progression in vivo.

In conclusion, we showed that EPA treatment inhibits the apoptosis of chondrocytes and expression of MMPs by oxidative stress in vitro, and prevents OA progression in vivo.
Significance: EPA as a medication or supplement could be a powerful tool for the prevention of OA progression.
Figure 3

(A)  (B)  (C)

(D)  (E)  (F)

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