NOTE *Surgery*

Suppressive Effect of Hyaluronan on Chondrocyte Apoptosis in Experimentally Induced Acute Osteoarthritis in Dogs

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ABSTRACT. Dogs receiving anterior cruciate ligament transection (ACLT) were treated with either intravenous (IV) or intraarticular (IA) administration of hyaluronan (HA), and differences in appearance of chondrocyte apoptosis of the stifle joint were investigated. Chondrocyte apoptosis was detected using flow cytometry as well as by staining with TdT-mediated dUTP nick end labeling (TUNEL). The percentage of apoptotic chondrocytes in dogs with ACLT was significantly higher than that in intact (non-ACLT) dogs. Dogs treated with IA or IV injection of HA after ACLT had fewer apoptotic chondrocytes than non-treated dogs after ACLT. It was suggested that ACLT-induced apoptosis of chondrocytes was suppressed by HA administration of either IA or IV.
KEY WORDS: apoptosis, chondrocyte, hyaluronan.

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Osteoarthritis (OA) or degenerative joint disease is a disease of the joint in which the articular cartilage degenerates and the synovial membrane becomes inflamed. OA is estimated to affect as much as 20% of the canine population over 1 year of age [11], and the incidence is thought to increase with age. Since OA lesions are usually irreversible, the goal of treatment for OA is to alleviate discomfort, prevent or at least retard further degenerative changes, and restore affected joints to as close to normal function as possible while minimizing pain [22].

Articular cartilages in human OA joints have more apoptotic chondrocytes than in normal joints, and apoptosis is considered to play an important role in the development of OA [10,13]. Articular cartilages in an experimental rabbit OA model induced by anterior cruciate ligament transection (ACLT) also showed more apoptotic chondrocytes than in normal rabbits [8]. It is known that ACLT is the model of acute phase in OA. In dogs, however, it is unclear whether or not the number of apoptotic chondrocytes increases by ACLT.

Hyaluronan (HA) is a major natural component of synovial fluid and the cartilage extracellular matrix. An intraarticular (IA) injection of HA is reported to be clinically effective in alleviating articular pain and can slow the degeneration of articular cartilage [1, 7]. The IA administration of HA is believed to compensate the deficient component of articular cartilage. However, recent studies have proven that HA also has some biochemical effects, such as inhibition of reactive oxygen production [6], coating of the pain receptors [18] and inhibition of prostaglandin E2 production [19]. From these findings, it is expected that the oral or intravenous (IV) administration of HA could also be effective as a treatment for OA. In fact, IV administration of HA improved the clinical signs of osteochondral fragmentation in horses [12]. In our preliminary research using sheep and dogs, IV administration of HA could suppress cartilage degeneration histopathologically (unpublished data).

The purpose of the present study was twofold: to investigate the change in apoptotic chondrocytes in dogs with an acute phase in OA induced experimentally by ACLT, and to evaluate the suppressive effect of IA or IV injection of HA on the progress of apoptosis of chondrocytes in the articular cartilage in dogs.

This study was conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. Twenty-nine Beagle dogs (20 males and 9 females) with a mean age of 14.9 (range, 11 to 27) months and a mean body weight of 9.1 (range, 6.2 to 13.0) kg were used in this study. They were randomly assigned into four groups. Five dogs in Group 1 (control) received neither surgery nor HA administration. Eight dogs in Group 2 underwent ACLT but did not receive HA. Sixteen dogs underwent ACLT and received either IA (Group 3, n=8) or IV (Group 4, n=8) administration of HA after ACLT.

Anterior cruciate ligament transection was performed on the right stifle joint. Midazolam (0.1 mg/kg) and butorphanol (0.2 mg/kg) were administered intravenously as preanesthetic medications. Anesthesia was induced using thiopental sodium (12.5 mg/kg) and maintained with isoflurane and oxygen.

The dog was positioned in left lateral recumbency, and the right knee was prepared for surgery. Through the lateral approach, the cranial cruciate ligament was exposed and severed by a No. 19 blade. After cranial drawer motion was confirmed, the joint was closed routinely. Subcutaneous injections of 0.2 mg/kg butorphanol for analgesia and 20 mg/kg cefazolin twice a day were continued for 3 days fol-

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lowing the surgery. The dogs were housed individually for 7 days after surgery. They were then allowed to exercise in a large yard once a day for 1 hr, during which time their levels of lameness were observed. All dogs in Groups 2, 3, and 4 were euthanized 12 weeks after ACLT.

HA (Hyonate®), supplied by Bayer Medical Co. (Tokyo, Japan), is produced from *Streptococcus sp.* with a molecular weight of 120–600 × 10^3. HA injection protocols were decided according to previous reports on horses [1, 6, 12, 21]. In Group 3, 1 m*l* (10 mg HA) was administered IA under sedation with medetomidine (20 μg/kg) and midazolam (0.3 mg/kg). In Group 4, 2 m*l*/head of HA was administered IV via the cephalic vein. The dogs in Groups 3 and 4 each received HA once per week for five consecutive weeks beginning on the day after surgery [21].

Blood examinations, including complete blood count, alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine, and electrolytes, were performed before and 6 and 12 weeks after ACLT.

At the end of the experiment, the dogs were euthanized by administration of an excess dose of thiopental. Within 2 hr after euthanasia, the articular cartilage tissues were obtained from the medial and lateral surfaces of the right distal femoral condyle (the operated stifle joint) of five dogs in each of Groups 2, 3, and 4 and from the same site of the intact left distal femoral condyle in all five dogs from Group 1. The propidium iodide (PI) method was used to quantify apoptotic chondrocytes [4]. PI fluorescence of nuclei was measured by flow cytometry on a fluorescence-activated cell sorter (FACSCalibur, Nippon Becton Dickinson, Tokyo, Japan). Data were expressed as the percentage of apoptotic (hypodiploid) nuclei.

In situ detection of apoptosis was performed on three of eight dogs in each of Groups 2, 3, and 4, and on all five dogs of Group 1. Medial and lateral condyles of the right femur, which had been subjected to ACLT in Groups 2, 3, and 4 and which was intact in Group 1, were used for this examination. After decalcification of the condyle, cartilage and subchondral bone sections were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 4-μm-thick sections were cut for *in situ* apoptosis detection under light microscopy. Apoptosis was detected using the Apoptosis *in situ* Detection Kit (Wako, Osaka, Japan) according to the manufacturer's instructions. This kit used the TdT-mediated dUTP nick end labeling (TUNEL) method. TUNEL-positive cells were counted under light microscopy. Apoptosis was expressed as the percentage of apoptotic nuclei/100 nuclei by counting no fewer than 400 cells from among four sites in the superficial and middle zones of the cartilage. Mean counts in the medial and lateral condyle were regarded as the values of individual samples. Hematoxylin and eosin (HE) staining was also performed for light microscopy.

Scheffe's test was used for statistical analysis. P values less than 0.05 were considered statistically significant.

The percentage of apoptotic cells in the cartilage of each group is shown in Fig. 1. The percentages of apoptotic cells

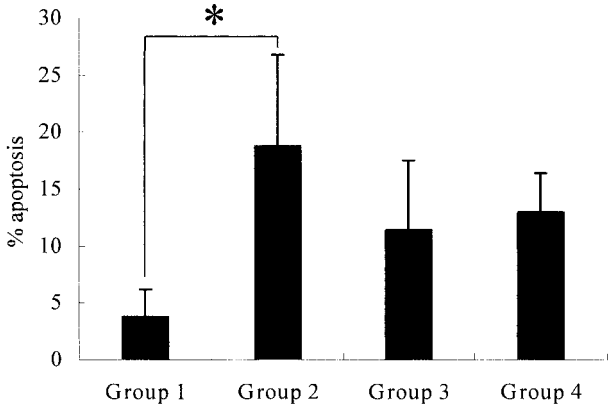

Fig. 1. The percentage of apoptotic chondrocytes detected by flow cytometry in each group. *: values were significantly different between the two ($p<0.05$).

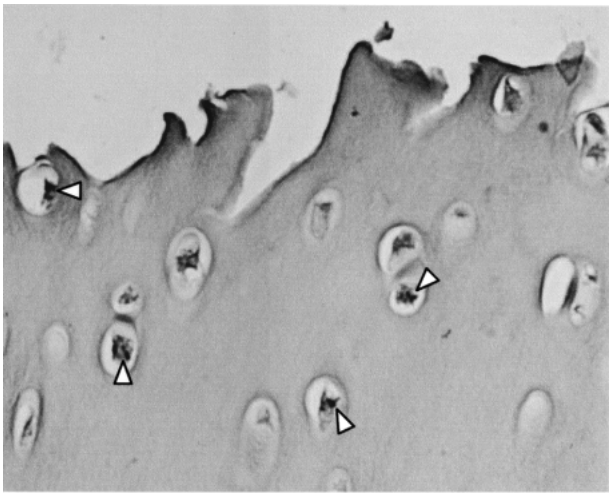

Fig. 2. The cartilage tissue of Group 2 (ACLT) showing apoptotic chondrocytes under TUNEL staining. TUNEL-positive cells (arrow head) were observed (× 400).

were 3.8 ± 2.4% in Group 1 (non-ACLT), 18.8 ± 8.0% in Group 2 (ACLT), 11.4 ± 6.2% in Group 3 (ACLT + HA-IA), and 13.0 ± 3.4% in Group 4 (ACLT + HA-IV). The value of Group 1 was significantly lower than that of Group 2, but there were no other significant differences between groups.

In the TUNEL-staining, apoptotic chondrocytes were seen in abundance in the cartilage samples of Groups 2, 3, and 4, while only a few were seen in those of Group 1. Figures 2 and 3 show an example of TUNEL-stained cartilage tissue, where apoptotic chondrocytes in the cartilage of a dog that received ACLT can be seen not only in the superficial and middle zones, but also in the area near the tidemark. Apoptotic chondrocytes were also confirmed in HE-stained cartilage tissues, where they had the typical condensed, pyknotic nuclei and deeply eosinophilic, shrunken cyto-

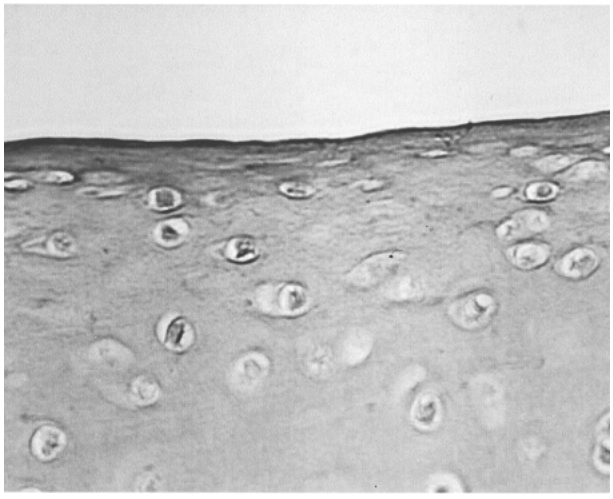

Fig. 3. The cartilage tissue of Group 1 (non-ACLT). No TUNEL-positive cells were observed in this area (× 400).

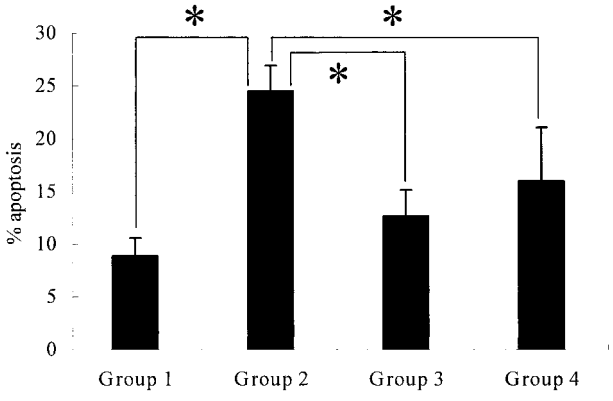

Fig. 4. The percentage of apoptotic chondrocytes detected *in situ*. *: values were significantly different within the groups ($p<0.05$).

plasm.

The percentages of TUNEL-positive cells were 8.9 ± 1.7% in Group 1, 24.5 ± 2.5% in Group 2, 12.7 ± 2.5% in Group 3, and 16.0 ± 5.1% in Group 4 (Fig. 4). The value of Group 2 was significantly higher than the others. There was no significant difference between Groups 3 and 4.

Although lameness scores were not recorded in this study, all dogs in Group 2 showed moderate lameness after ACLT through the end of this study. Some dogs in Groups 3 and 4 sometimes showed mild lameness, but the others in those groups did not. On blood examinations, all dogs tested were within normal ranges.

Recent reports have found many apoptotic chondrocytes in the cartilage of human OA patients [2, 10, 13]. It has also been found that the rabbit OA model with ACLT had an increased number of apoptotic chondrocytes [8]. From these findings, it is believed that apoptosis of chondrocytes is the initial change of the OA lesion. Chondrocyte apoptosis alters the cartilage matrix synthesis, leading to matrix degeneration and destruction, and finally to OA.

In this study, using ACLT dog model, the percentage of apoptotic chondrocytes in Group 2 was 18.8 ± 8.0% on flow cytometry and 24.5 ± 2.5% on *in situ* detection of apoptosis. These values were higher than those of Group 1, and the difference was statistically significant. These results suggested that chondrocyte apoptosis was increased in the ACLT dogs.

Because the anterior cruciate ligament contributes to the stability of the stifle joint, the articular cartilage will receive an abnormally higher load due to the joint instability resulting from ACLT. In previous reports, pressure to the cartilage induced chondrocyte apoptosis [5, 16]. Although the entire apoptotic cascade is still not fully understood, nitric oxide (NO) produced in the cartilage tissue and inflammatory mediators from the dissected synovial membrane have been linked to chondrocyte apoptosis [3, 8, 9, 13]. NO is one of the molecules that trigger cartilage breakdown via activating matrix metalloproteinases activity. This study did not clarify the mechanism underlying chondrocyte apoptosis, but it did demonstrate that experimental ACLT might increase chondrocyte apoptosis in dogs.

In this study, the percentages of chondrocyte apoptosis in Groups 3 and 4 were significantly lower than that in Group 2 according to *in situ* detection. On flow cytometry, however, there was no statistically significant difference between Group 2 and Groups 3 and 4, though the percentages in Groups 3 and 4 were lower than that in Group 2. This may indicate that HA administration via either IA or IV route could suppress chondrocyte apoptosis and thus could be an alternative therapy for canine OA.

In the synovial fluid of OA, the amount of HA and its viscosity may decrease [20]. Initially, an intravenous HA administration was expected to increase its contents within the joint capsule, to normalize the condition of the synovial fluid, and to protect cartilage physically as a form of viscosupplementation [17]. In fact, an IV injection of HA was clinically effective for treating OA in humans and horses [1, 7]. HA has been found to have many biochemical properties, such as inhibition of reactive oxygen production [6], coating of the pain receptors [18], and inhibition of prostaglandin E2 production [19]. It is not clear why the IV injection of HA suppressed apoptosis of chondrocytes in this study. However, further studies should be undertaken to clarify the mechanism by which HA protects chondrocytes. In addition, IV administration is a much simpler procedure than IA administration.

In conclusion, the results of this study suggested that chondrocyte apoptosis was increased in dogs that had received ACLT. Both IA and IV administrations of HA effectively decreased chondrocyte apoptosis.

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