Effect of Intra-articular Injection of AOD9604 with or without Hyaluronic Acid in Rabbit Osteoarthritis Model

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Abstract. Objective. To investigate the effects of AOD9604 intra-articular injections with or without hyaluronic acid (HA) in a collagenase-induced knee osteoarthritis (OA) rabbit model. Design. Mature New Zealand white rabbits (n=32) were randomly administered 2 mg collagenase type II twice in each knee joint. Weekly injections of 0.6 mL saline (Group 1), 6 mg HA (Group 2), 0.25 mg AOD9604 (Group 3), and 0.25 mg AOD9604 with 6 mg HA (Group 4) were administered for 4-7 weeks after the first intra-articular collagenase injection. The degree of cartilage degeneration was assessed using morphological and histopathological findings, and the degree of lameness was observed at 8 weeks after the first collagenase injection. Results. Mean gross morphological and histopathological scores were significantly higher in Group 1 than in Groups 2, 3, and 4, and the scores were significantly lower in Group 4 than in Groups 2 and 3. The lameness period in Group 4 was significantly shorter than those in Groups 1, 2, and 3. The lameness period in Group 1 was significantly longer than those in Groups 2 and 3. Conclusion: Intra-articular AOD9604 injections using ultrasound guidance enhanced cartilage regeneration, and combined AOD9604 and HA injections were more effective than HA or AOD9604 injections alone in the collagenase-induced knee OA rabbit model.

Key words: Osteoarthritis, Knee, AOD9604, HA, Collagenase, Ultrasound.

Introduction

Osteoarthritis (OA) is a degenerative joint disease that results from articular cartilage loss induced by complex interactions of genetic, metabolic, biochemical, and biomechanical factors with secondary components of inflammation [1]. OA is the most common arthritis and a major medical problem in people aged 65 years and older [2]. Non-steroidal anti-inflammatory drugs (NSAIDs), physical therapy, exercises, corticosteroids, and hyaluronic acid injections have all been used for the conservative treatment of OA. The role of NSAIDs in OA management is controversial because of its adverse side effects, as well as side effects on the cartilage [3].

Recent advances in the field of regenerative medicine, such as the use of platelet-rich plasma and stem cell injections, are emerging as the preferred options for treating OA. This is in part because patients do not desire only temporary alleviation of symptoms. Rather, patients also seek permanent correction and repair of the underlying biology for regenerating the damaged tissue in order to permanently alleviate their symptoms [4]. The aforementioned treatment options have been used in several areas of medicine for delivering growth factors to optimize healing.

Cartilage loss in OA is caused by proteoglycan depletion and chondrocyte death that in turn are caused by imbalances between catabolic and anabolic activities within the joint [5]. Growth hormone (GH) has been shown to correct this imbalance [6]. Although the exact mechanism underlying the effects of intra-articular GH injection is not known, GH in the synovial fluid probably enhances proliferation, matrix synthesis, and differentiation of bone and cartilage cells in vitro [7]. Studies have found that GH accelerates healing in animal models of OA [8,9]. However, intra-articular GH injections in humans are known to have detrimental pro-tumor and pro-diabetic effects. These negative effects are caused by the secondarily produced insulin-like growth factor-1 (IGF-1) [10].

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AOD9604 is a new synthetic peptide fragment that comprises a modified 15 amino acid region of GH with a tyrosine component to help stabilize the molecule. Similar to GH, AOD9604 aids weight reduction in rodent models of obesity and was originally developed for the treatment of obesity in humans [11]. Additionally, it does not stimulate the production of IGF-1 [10], has positive effects on the differentiation of adipose mesenchymal stem cells into bone, and was found to promote proteoglycan and collagen production in isolated bovine chondrocytes in an in vitro study by Metabolic Pharmaceuticals (patent applied [WO2013082667]). Its positive effects include promoting the repair of bone and cartilage in cases of OA.

The aim of this study was to investigate the protective effects of AOD9604 intra-articular injection on the cartilage in a collagenase-induced knee OA rabbit model. The efficacy of ADO9604 combined with hyaluronic acid (HA) injection was also evaluated.

Materials and Methods

Animals. Male New Zealand white rabbits (n=32; aged 12 weeks) were used in the experiments. Animals were housed in separate metal cages at a temperature of 23°C±2°C and relative humidity of 45%±10%. The animals were allowed free access to tap water and were fed a commercial rabbit diet. Animal experiments were performed in accordance with internationally accredited guidelines and approved by laboratory’s Institutional Animal Care and Use Committee.

Experimental OA model. Experimental animals were anesthetized with an intramuscular injection of xylazine (Rompun®; Bayer Co., Seoul, Korea) at a dose of 1.9 mg/kg and ketamine (Ketar®; Yuhan Co., Seoul, Korea) at a dose of 46 mg/kg. The right knee joints of all rabbits were shaved and sterilized. Then, 2 mg of collagenase type II from Clostridium histolyticum (Sigma Co., St. Louis, MO, USA) was prepared for intra-articular injection. Collagenase was dissolved in a sterile phosphate buffered saline (pH 7.4) and filtered with a 0.22-μm membrane. The solution was slowly injected into the right knee joint using ultrasound guidance. The same injection was repeated 3 days after the first injection, as previously reported (Figure 1) [8].

Drug administration. Thirty-two rabbits were divided into 4 equal groups. Four different solutions, including saline, HA, AOD9604, and AOD9604 with HA, were injected in each group on a weekly basis for 4-7 weeks after the first collagenase injection. Group 1 received intra-articular saline injection (0.6 mL). Group 2 received intra-articular HA, (Hyruan-plus®; LG Life Science, Daejeon, Korea) injection (6 mg). The molecular weight of HA was measured at 3.0×10^6 Da, and it was prepared to a 10 mg/mL concentration. Group 3 received intra-articular AOD9604 (Metabolic Pharmaceuticals, Melbourne, Australia) injection (0.25 mg per 0.6 mL). Group 4 received combined intra-articular AOD9604 (0.25 mg) and HA (6 mg) injections. All injections were administered by a physiatrist, using a commercially available ultrasound system with 3–12 MHz multi-frequency linear transducer (E-CUBE 15®; Alpinion Medical Systems, Seoul, Korea) under general anesthesia and under sterile conditions (Figure 1).
medication was administered after the injection. The rabbits were euthanized by CO inhalation 9 weeks after the first collagenase injection (Figure 2).

Lameness of the affected limb. The rabbits were clinically observed daily at 14:00. The rabbits were placed on a 2-m² ground area, and gait was individually assessed by direct observation for 20 minutes. The knee and ankle of the intact rabbit limb showed typical flexion and extension cycle during hopping. Lameness was defined as the inability to bear weight and the loss of typical flexion and extension cycle of the affected limb during hopping compared with that of the unaffected limb. The severity of lameness was not quantified. The time taken to return to normal ambulation without lameness of the affected limb was recorded for each group. The lameness period was checked by three independent physiatrists who did not have knowledge of the experimental groups.

Gross morphological examination. The knee joints were dissected after euthanasia. The medial and lateral condyles of the femur and tibia were examined for gross morphological changes. The degree of cartilage degeneration on the lateral part of the femoral condyle was assessed using the scoring system devised by Yoshimi et al. [12]. This area was assessed because the intra-articular collagenase injections had caused most damage at this area [13]. Normal cartilage was scored as 0, softened cartilage as 1, fibrillated cartilage as 2, eroded cartilage as 3, ulcerated cartilage as 4, and loss of cartilage as 5.

Histopathological examination. The lateral and medial condyles of the femur and tibia were fixed with 10% neutral buffered formalin and decalcified with 20% ethylenediaminetetraacetic acid (EDTA). Calcified condyles were embedded in paraffin, and standard frontal sections of 5 µm were prepared and stained with haematoxylin and eosin in the cartilage of the lateral part of the femoral condyle, according to gross morphological observations [14]. If the staining was not adequate, the specimen was cut at the next cartilage surface. Cartilage degradation features were analyzed using the scoring system modified by Mankin et al. [14]. Histopathological evidence of cartilage degeneration was evaluated by structural scoring (0, normal; 1, surface irregularities; 2, pannus and surface irregularities; 3, clefts to transitional zones; 4, clefts to radial zones; 5, clefts to calcified zones; and 6, complete disorganization) and cell status (0, normal; 1, diffuse hypercellularity; 2, cloning; and 3, hypocellularity) of the articular cartilage. Total score ranged from 0 (normal) to 9 (complete disorganization and hypocellularity of the articular cartilage). All sections were graded by two independent pathologists who did not have any information about the injection solutions.

Statistical analysis. Statistical analysis was performed using SPSS version 14.0 (SPSS, Chicago, Ill). The differences of gross morphological and histolopathological findings and lameness period among four groups were assessed using the Kruskal–Wallis test. The Mann–Whitney U test was used to compare the gross morphological and histolopathological findings and lameness period between two groups, and p-values <0.05 were considered statistically significant.

Results

Gross morphological and histopathological findings according to four different injections. The four groups showed different gross morphological damage and histopathological changes in the cartilage of the lateral part of the femoral condyle (Figure 3). Complete disorganization of articular cartilage with apparent cloning of chondrocytes in the transitional and radial zones was evident in Group 1 (Figures 3-A,E,I). Abnormal gross morphological and histopathological changes such as fibrillated and irregular cartilage surfaces, disappearance of surface-layer cells, and slightly diffused cell growth in the transitional and radial zones were observed in Group 2 (Figures 3-B,F,J). Erosion of the articular cartilage, cleft, and cell cloning in the transitional and radial zones were noted in Group 3 (Figures 3-C,G,K). Softening of articular cartilage and surface irregularities were noted in Group 4 (Figures 3-D,H,L).
AOD9604 effect in rabbit osteoarthritis

Mean gross morphological and histopathological scores in Group 1 were significantly higher than those in Groups 2, 3, and 4 ($p<0.05$). Mean gross morphological and histopathological scores in Group 4 were significantly lower than those in Groups 2 and 3 ($p<0.05$). However, there are no differences between the mean gross morphological and histopathological scores of Groups 2 and 3 (Figures 4 and 5).

**Lameness period.** The mean time (± SD) taken for recovery of normal ambulation was 25±2 days in Group 1, 15±3 days in Group 2, 16±2 days in Group 3, and 11±4 days in Group 4. The lameness period in Group 4 was significantly shorter than those in Groups 1, 2, and 3 ($p<0.05$). The lameness period in Group 1 was significantly longer than those in Groups 2 and 3 ($p<0.05$). However, there were no differences in the mean lameness period between Groups 2 and 3 (Figure 6).

**Discussion**

We found that osteoarthritisic rabbits administered intra-articular AOD9604 injections had better outcomes with lesser morphological and histopathological damage than was observed in the control group. AOD9604 is a disulphide-constrained peptide that comprises 15 amino acids from the C-terminal sequence of human GH and an additional N-terminal tyrosine residue: YLRIVQCRSVEGSCGF [15]. The exact mechanism underlying the action of GH in OA is unknown. Previous studies have shown that GH can act directly on the growth plate by stimulating local production of IGF-1 and by increasing cartilage metabolism [9,16] and chondrocyte proliferation [17]. Although AOD9604 is not a high-affinity agonist of the GH receptor and does not stimulate the proliferation of cells transfected with the GH receptor, it retains some functions of GH [11].
Initially, AOD9604 was investigated for the treatment of obesity in humans. In rodent models of obesity, AOD9604 showed a similar effect of weight loss as that observed with GH [11]. However, AOD9604 does not induce diabetes and does not stimulate the production of IGF-1 [10].

*In vitro* studies by Metabolic Collaborators showed that AOD9604 enhances the differentiation of adipose mesenchymal stem cells into bone, promotes proteoglycan and collagen production in isolated bovine chondrocytes, and promotes differentiation of myoblasts into C2C12 cells. These effects induced by AOD9604 are similar to those required for the repair of bone, cartilage, and muscle, all of which are affected in OA. To the best of our knowledge, no study has compared the effects of GH and HA intra-articular injections on OA. A previous study showed the effects of intra-articular GH injection on articulophyseal cartilage regeneration in the knees of rabbits [9]. Our study showed that the groups that received AOD9604 or HA injection had better outcomes in terms of morphological and histolopathological findings, as well as a lowered duration of lameness than the group that received saline injections, although there are no significant differences between the two groups. In addition, our study revealed that the groups that received combined injections of AOD9604 and HA showed better outcomes than the groups that received AOD9604 or HA alone. The apparently synergistic effect of combined injections is thought to indicate that intra-articular injection of HA may have a chondrocyte-protective role, and the AOD9604 could help recapitulate the developmental cascades which regrows a segment of the articular cartilage in a joint. Our results are consistent with those of a previous study [8] that combined the injection of HA with recombinant human GH and found that the combination is more effective than HA injection alone.

In this study, AOD9604 was given in a dose of 0.25 mg that is comparable to the dose used in a previous report [8] on GH in promoting recovery to normal walking and in joint repair in the rabbit collagenase model of osteoarthritis. AOD9604 is a fragment of GH; therefore the dose of AOD9604
used was the molar equivalent of the active GH dose that the previous study [8] used. Human GH was given as 3 mg in 0.6 ml intra-articular injection volume. On a molar basis, 3 mg of GH equates to 0.25 mg of AOD9604. In addition, published data [10] suggest that the volume of synovial fluid in an arthritic rabbit is approximately 0.7 ml. Combined with the injection volume, this gives a total volume of 1.3 ml and therefore an initial concentration of AOD9604 of 0.19 mg/ml. In a previous study [11] of GH in the beagle after intra-articular injection, researchers injected 1.5 mg of GH in aqueous solution in 0.15 ml volume. The aqueous formulation gave an initial concentration of approximately 200-300 ug/mL in the synovial fluid. On a molar equivalent basis this equates to 0.11 mg/mL of AOD9604, which is close to the value used in this study.

The particle size of a drug molecule is a crucial factor in the effects of intra-articular injections. The larger the particle size, the longer the drug will stay in a joint, thereby increasing its efficacy [20]. The short residence time of intra-articular AOD9604 when dissolved in saline was due to its rapid uptake by local circulation. However, the combination of AOD9604 with HA may result in more residence time and better effects in the joint. Recent studies [21-23] have shown that the addition of HA to nanoparticles improves the drug effects by increasing drug bioavailability and decreasing systemic absorption after topical administration.

The high molecular weight of HA significantly protects chondrocytes against oxygen-derived free radical action [24]. In our study, high molecular weight HA was injected in combination with AOD9604 and may have resulted in a synergistic effect on proteoglycan and collagen production in chondrocytes and on chondrocyte protection in OA.

Acromegaly is characterized by an excessive amount of articular cartilage in joints caused by excess GH secretion [25]. The tremendously thick articular cartilage in acromegaly can be explained by the local production of IGF-1 in cartilage cells through GH receptors [9,18]. Long-term treatment with GH might induce hypertrophy of the cartilage and changes in the joint geometry because of altered subchondral bone structures. Long-term treatment with GH by local injections may also be associated with various risks, including glucose intolerance, insulin resistance, diabetes, cancer, edema, and hypertension [26-29]. AOD9604 is not an agonist with a high affinity to the GH receptor and does not stimulate the production of IGF-1. Therefore, AOD9604 may be safer than human recombinant GH for the long-term treatment of OA.

In our study, the rapid recovery from lameness (11 days) in the group that received AOD9604 and HA injection suggests that an early anti-inflammatory and pain-relieving effect could be induced before the tissue repair observed at the end of the treatment period (35 days). This result may be explained by the pain-relieving effects of GH [30]. The intra-articular injection to the human knee using ultrasound guidance notably enhances the accuracy compared with injection using anatomical guidance [31-33]. Until recently, intra-articular injections to the rabbit knee using ultrasound guidance have rarely been reported [34]. In our study, intra-articular injections were performed using ultrasound guidance to identify the correct trajectory for needle placement in the knee joint, as the rabbit knee joint is smaller than that of humans.

It is not advisable to generalize our results for the OA in a rabbit model because of the small sample size of this study. Further studies with larger sample sizes and longer follow-ups are necessary to establish the validity of our results. Moreover, the different effects caused by varying intra-articular dosages, formulations, and injection intervals need to be assessed.

In conclusion, intra-articular AOD9604 injections using ultrasound guidance enhanced cartilage regeneration, and combined AOD9604 and HA injections were more effective than HA or AOD9604 injection alone in the collagenase-induced knee OA rabbit model.

Acknowledgment

We would like to thank Dr. Andrew Gearing for his help in preparing the manuscript.

References

International Association for the Study of Obesity 2006;7:239-250.