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Note

Effects of Pro-Hyp, a Collagen Hydrolysate-Derived Peptide, on Hyaluronic Acid Synthesis Using *in Vitro* Cultured Synovium Cells and Oral Ingestion of Collagen Hydrolysates in a Guinea Pig Model of Osteoarthritis

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Proline-hydroxyproline (Pro-Hyp) stimulated hyaluronic acid production in cultured synovium cells. It was detected in guinea pig blood after oral ingestion of collagen hydrolysates. Oral administration of collagen hydrolysates increased the amount of proteoglycans in the epiphyses. It also reduced the morphological changes associated with osteoarthritic cartilage destruction of the knee joint. The results suggest that collagen hydrolysates have therapeutic potential for treatment of osteoarthritis.

Key words: proline-hydroxyproline (Pro-Hyp); hyaluronic acid; collagen hydrolysates; osteoarthritis; proteoglycan

Collagen is a major constituent of connective tissues in mammals, birds, and fishes. It has a unique triple helix configuration with a repeating amino acid sequence, (Gly-X-Y)_n, where X and Y are typically proline and hydroxyproline (Hyp) respectively.^{1,2)} Gelatin, a denatured form of collagen, is prepared on an industrial scale from animals.³⁾ Gelatin-based food derivatives obtained from animals, especially fish and porcine, have attracted much attention as health-food ingredients worldwide.

We have reported that several food-derived collagen peptides were detected in human blood 2 h after oral ingestion of fish scale and porcine skin collagen hydrolysates.⁴⁾ The major constituent of the food-derived collagen peptides that remained in the blood was identified as Pro-Hyp. Recently, we indicated that Pro-Hyp enhances both cell mitotic activity and hyaluronic acid synthesis in human dermal fibroblast cells, which is mediated by the activation of hyaluronan synthase 2 (*HAS2*) transcription.⁵⁾ Thus, it can be assumed that food-derived collagen peptides in the blood are involved in some of the biological activities suggested by animal and human experiments.

Osteoarthritis (OA) is a degenerative joint disease that is a major cause of disability in humans. Aging, mechanical stress, traumatic injury, genetic susceptibility, and metabolic predispositions are considered risk factors for the disease.⁶⁾ Message expression of *HAS1* and *HAS2* in the synovium of OA is significantly less

than in the healthy control synovium, whereas that of hyaluronidase 2 in the synovium of OA is significantly greater than in the healthy control synovium. Decreased expression of *HAS1* and *HAS2* message and/or increased expression of the message for hyaluronidase 2 is reflected in a reduced concentration and decreased average molecular weight of hyaluronic acid in the joint fluids of patients with OA.⁷⁾

Here we studied the effects of Pro-Hyp on glycosaminoglycans synthesis using *in vitro* cultured synovium cells and oral ingestion of collagen hydrolysates in a guinea pig model of OA.

Synovium cell line HIG-82 (Dainippon Sumitomo Pharma, Osaka, Japan) was used. Cells were grown at 37 °C in 35-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen) in a humidified atmosphere of 5% CO₂/95% air. The cells were grown to confluency in 10% FBS-DMEM and then placed in serum-free DMEM for 24 h. They were treated with Pro-Hyp (Bachem, Budendorf, Germany) (50 µg/ml) for the last 48 h of treatment. The contents of hyaluronic acid in medium were detected by the method of Hata and Nagai.⁸⁾ PGs in the medium was solubilized in 0.5 M NaOH. Glycosaminoglycans were released from the PGs at this step. The sample was adjusted to pH 7.8 with 1 N HCl, and then it was recombined and subjected to pronase digestion in 0.1 M Tris-HCl (pH 7.8) containing 5 mM CaCl₂ at 50 °C overnight. It was deproteinized by adding pronase. The digest was TCA precipitated on ice, centrifuged, and the acid soluble glycosaminoglycans were dialyzed extensively against H₂O. Glycosaminoglycans were electrophoretically resolved on a cellulose acetate membrane. The Alcian Blue staining band intensities of these digital images were determined using Quantity One imaging software.

Collagen hydrolysates were derived from fish scale type I collagen and porcine skin type I collagen (Nitta Gelatin, Osaka, Japan). Retired male breeder Dunkin-Hartley guinea pigs (SLC, Shizuoka, Japan) aged 12-months or older were used. Dunkin-Hartley guinea pigs display spontaneous onsets of progressive changes in the knee joints that closely resembles the develop-

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Abbreviations: Hyp, hydroxyproline; Pro-Hyp, proline-hydroxyproline; OA, osteoarthritis; PGs, proteoglycans

ment of OA in humans.^{9,10} All the animal experiments in this study were carried out in accordance with the ethics guidelines established by the Animal Committee of Meiji Seika Kaisha, Ltd., Food & Health R&D Laboratories. The guinea pigs (1.08 ± 0.02 kg body weight) were randomly placed into three treatment groups: group 1 ($n = 12$) control (distilled water); group 2 ($n = 12$) fish scale type I collagen hydrolysates; and group 3 ($n = 10$) porcine skin type I collagen hydrolysates. Each collagen hydrolysates preparation was suspended in 0.84 g/5 ml of distilled water. The guinea pigs were orally administered the various collagen hydrolysate suspensions at a dose of 0.84 g/kg/d (5 d per week) for 4 weeks. On the day of sacrifice, blood samples were obtained under ether anesthesia *via* the abdominal vein 2 h after ingestion, and were collected in a tube containing a final concentration of 4 mM EDTA. Plasma was prepared and was stored at -80°C until use. After blood sampling, the guinea pigs were euthanized by an overdose of ether. The left knee joints were removed and fixed in a 10% neutral-buffered formalin solution (Wako, Osaka, Japan). The right knee joints were removed and analyzed for PG content.

Histological grading was performed on coronal sections of cartilage from the damaged area of the left tibial plateau. Specimens were dissected, fixed in a 10% neutral-buffered formalin solution for 5 d, subjected to decalcification in a 10% formic acid-formalin solution for 10 d, and embedded in paraffin for histological evaluation. Serial sections ($4\ \mu\text{m}$) were stained with hematoxylin and eosin or Safranin O-fast green. The severity of the OA lesions was graded on a scale of 0–14 by one blinded observer, using the criteria of Mankin *et al.*¹¹

The plasma was de-proteinized by adding an equal volume of 5% (w/v) TCA. Pro-Hyp analysis was performed by LC-MS/MS analysis by the method of Ichikawa *et al.*¹²

PGs analysis was performed by western blotting. Epiphyses from the right tibia were dissected and defatted with hexanes. The samples were ground in liquid nitrogen with a refrigerated mill (Bio Medical Science, Tokyo, Japan). Bone matrix proteins were extracted at 4°C with 4 M guanidine HCl containing a protease inhibitor consisting of 0.1 M 6-aminohexanoic acid, 10 mM EDTA, and 5 mM benzamidine HCl.¹³ The extracts were dialyzed against 5 mM Tris-HCl (pH 7.4) containing 7 M urea. Aliquot of nondialyzable materials were purified using a batch system with a DEAE-Toyoperal 650S gel. The absorbed fraction was eluted with 5 mM Tris-HCl containing 7 M urea, 2 M NaCl, and protease inhibitors. The protein concentration in the eluted sample was determined by the method of Lowry. The sample ($12\ \mu\text{g}$ protein) was used for western blotting of PGs. Anti-PG Δ Di-6S (Seikagaku, Tokyo, Japan) was used as the primary antibody. The secondary antibody consisted of anti-mouse IgG HRP conjugates (Promega, Madison, WI). Bound antibodies were visualized using the ECL kit (GE Healthcare, Little Chalfont, England).

Results were expressed as mean \pm standard error (SE). Data were analyzed by the Wilcoxon rank sum test (comparison for Mankin score), Student's *t*-test (comparison for hyaluronic acid synthesis) and one-way

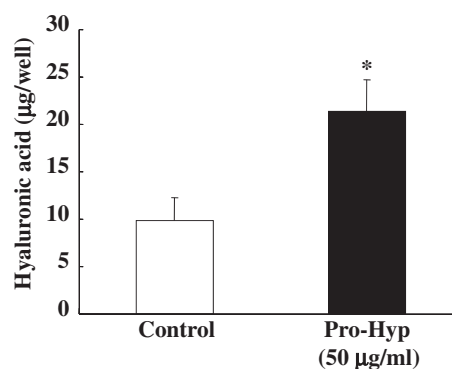


Fig. 1. Hyaluronic Acid Synthesis Was Increased by Pro-Hyp.

Synovium cells treated with $50\ \mu\text{g/ml}$ Pro-Hyp for 48 h. Data are presented as the mean \pm SE, $n = 3$. *Statistical significance at $p < 0.05$.

ANOVA followed by the *post-hoc* procedure of the Tukey-Kramer test (comparison for Pro-Hyp and proteoglycan) using StatLight software (Yukms, Tokyo, Japan).

Recently, we reported that Hyp-containing peptides were detected in human blood after oral ingestion of collagen hydrolysates from fish scales.⁴ The major constituents of Hyp-containing peptides that remained in the blood were identified as Ala-Hyp, Pro-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Phe-Hyp, Pro-Hyp-Gly, Gly-Pro-Hyp, Ile-Hyp, and Leu-Hyp. Moreover, Pro-Hyp was the major Hyp-containing peptide in human blood after oral ingestion of fish scale collagen hydrolysates, and Pro-Hyp reached a maximum concentration in human blood 2 h after oral ingestion of fish scale collagen hydrolysates.¹¹ Hence we studied Pro-Hyp effects on glycosaminoglycan synthesis using *in vitro* cultured synovium cells. Treatment of cultured cells with $50\ \mu\text{g/ml}$ Pro-Hyp for 48 h increased hyaluronic acid synthesis (medium) approximately 2.0-fold (Fig. 1). Thus Pro-Hyp enhanced hyaluronic acid synthesis in cultured synovium cells. On the other hand, synthesis of the other glycosaminoglycans did not change. Our study indicated that Pro-Hyp stimulated cell proliferation, *HAS2* mRNA expression, and hyaluronic acid production in human dermal fibroblasts.⁵ Moreover, Nakatani *et al.* indicated that Pro-Hyp enhanced chondrogenic cell differentiation.¹⁴ These results suggest that Hyp-containing peptide in the blood has biological activities *in vivo*/in joints, and so on.

Pro-Hyp in the guinea pig blood after ingestion of collagen hydrolysates was measured in order to determine whether Pro-Hyp was present in guinea pig blood. The amount of Pro-Hyp in the blood 2 h after ingestion of collagen hydrolysates is shown in Fig. 2A. The amount of Pro-Hyp in the fish scale collagen hydrolysates group was significantly different than in the control group. On the other hand, the amount of Pro-Hyp in the porcine skin group was not significantly different than in the control group. However, the amount of Pro-Hyp in the porcine skin groups was higher than in the control group. These results indicate that Pro-Hyp may indeed be transmitted to the tissue from circulating blood in guinea pigs.

Many hyaluronic acid chains bind to a core protein, resulting in large PGs. It has been reported that 12-

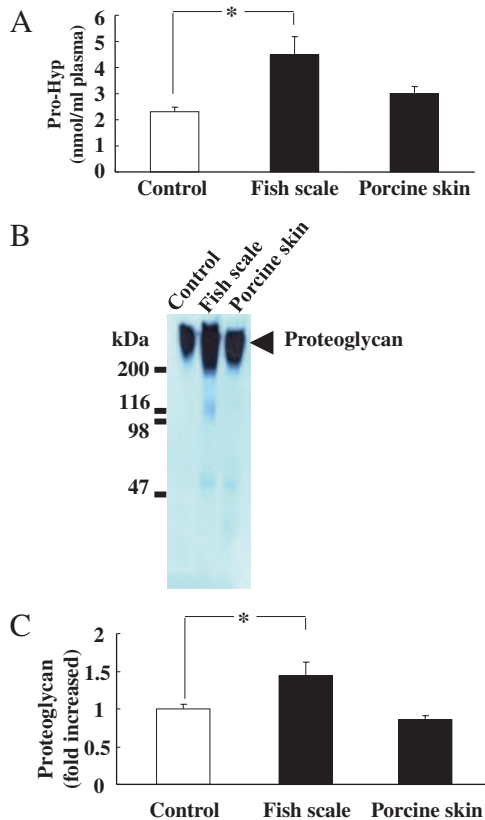


Fig. 2. Analysis of Pro-Hyp in Guinea Pig Blood (panel A), PGs in the Epiphyses of the Right Tibia (panel B), and Signal Intensities of PGs (panel C).

Analysis of Pro-Hyp in the blood 2 h after ingestion of collagen hydrolysates analysis was performed by LC-MS/MS analysis (panel A). Data are presented as the mean \pm SE, $n = 10-12$. *Statistical significance at $p < 0.05$. The content of PGs in extracts from the right tibia were analyzed by western blotting (panel B). The band intensities of these digital images were determined using Quantity One of imaging software for western blotting (panel C). Data are presented as the mean \pm SE, $n = 6-10$. *Statistical significance at $p < 0.05$.

month old guinea pigs with advanced OA had much lower amounts of PGs in the articular cartilage of the tibia condyles.¹⁵⁾ Hence, we determined PGs as a marker of OA in the guinea pigs. The content of PGs in the epiphyses was used as the amount of admixture from un-mineralized tissue for western blotting analysis (Fig. 2B and C). The amount of high molecular weight PGs ($>200,000$ MW) also increased. The amount of PGs in the fish scale collagen hydrolysates group was significantly different than in the control group, and was comparable to the increased Pro-Hyp levels in blood (Fig. 2A). Thus oral administration of collagen hydrolysates increased the amount of PGs in the epiphyses.

Figure 3 shows a histological grading of the tibial plateaus of the guinea pigs treated with control, fish scale, and porcine skin collagen hydrolysates. The tibia cartilage of the control group (Fig. 3B, left panel) showed severe reduction of the superficial zone (Safranin-O-staining) and clefts to calcified zone (structure). The tibia cartilage of the fish scale group (Fig. 3B, middle panel) and porcine skin group (Fig. 3B, right panel) showed moderate reduction of the superficial zone (Safranin-O-staining) and clefts to transitional zone (structure). The Mankin scores for the control group, the fish scale group, and the porcine skin group were $9.1 \pm$

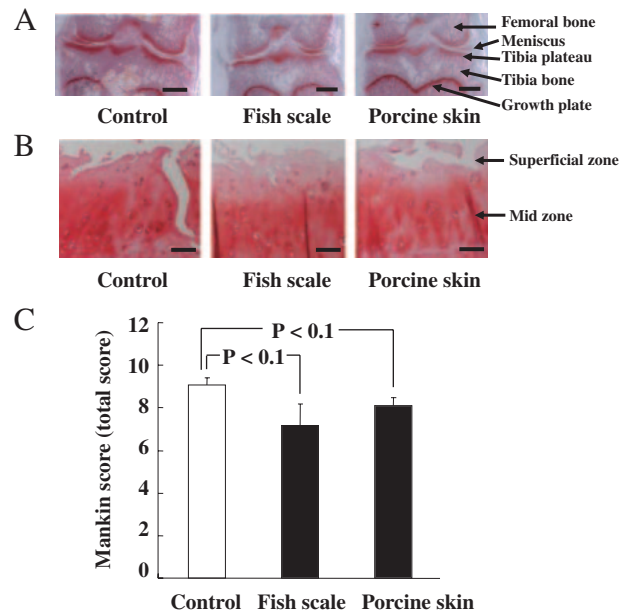


Fig. 3. Histological Grading of the Tibial Plateaus of Control, Fish Scale, and Porcine Skin Collagen Hydrolysates-Treated Guinea Pigs.

Control, fish scale, and porcine skin collagen hydrolysates were orally administered to guinea pigs for 4 weeks (5 d per week). Photomicrographs of guinea pig knee joints stained with Safranin O-fast green (panel A). Scale bar, 1 mm. Higher magnification of tibia plateau in the animal shown in panel A (panel B). Scale bar, 100 μ m. The severity of the tibial plateau was graded using the histological criteria of Mankin (panel C). Data are presented as the mean \pm SE, $n = 10-12$.

0.34 (mean \pm SE), 7.2 ± 1.03 , and 8.1 ± 0.38 , respectively (Fig. 3C). The scores for the fish scale ($p < 0.1$) and porcine skin groups ($p < 0.1$) tended to be lower than that of the control group.

Pro-Hyp concentrations in the blood, and the amount of PGs in the epiphyses for the fish scale collagen hydrolysates group, were significantly higher than the control group. On the other hand, the Mankin score for the fish scale collagen hydrolysates groups tended to be lower only than that of the control group, but this difference was not significant. These discrepancies may have occurred because an ingestion period for collagen hydrolysates of one month is too short to evaluate the efficacy of collagen hydrolysates in treating guinea pigs with advanced OA. Longer ingestion periods of collagen hydrolysate might yield more consistent results.

In summary, we found that Pro-Hyp stimulated hyaluronic acid production using *in vitro* cultured synovium cells. In a guinea pig model of OA, the amount of PGs in the epiphyses of the fish scale collagen hydrolysates group was significantly different than that of the control group, which was comparable to the increase of Pro-Hyp levels in the blood. Moreover, oral administration of collagen hydrolysates reduced the morphological changes associated with cartilage destruction of knee joint in a guinea pig model of OA. These results suggest that food-derived Hyp-containing peptides can affect the PGs and morphological changes associated with osteoarthritic cartilage, which might be mediated by stimulation of hyaluronic acid production in the synovium.

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