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Effects of Ingestion of Collagen Peptide on Collagen Fibrils and Glycosaminoglycans in Achilles Tendon

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Summary In order to investigate whether the oral ingestion of collagen peptide affects the extracellular matrix of tendon, two doses (0.2 g/kg and 1.0 g/kg body weight) were orally administered daily for 56 d to a rabbit, and both the size of collagen fibrils and the amount of glycosaminoglycans in the Achilles tendon were measured in comparison with those in a rabbit fed with a control protein, lactalbumin, or water alone. Ingestion of collagen peptide or lactalbumin induced a significant increase in collagen fibril diameter and a decrease in fibril density except for a high dose of lactalbumin compared with the water control. A histogram pattern of fibril diameter in a high dose of collagen peptide showed a peak at 160–180 nm, which was not observed in other groups. However the percentage of diameters over 200 nm was the lowest in this group but highest in the low-dose group of collagen peptide. The mean fibril diameter and mass average diameter of a high dose of collagen peptide were significantly smaller than those in a low dose. The amount of dermatan sulphate increased in the high-dose groups, while the amount of hyaluronic acid decreased in rabbits fed with collagen peptide or lactalbumin at either dose. These results suggest that the ingestion of collagen peptide affects the size of collagen fibrils and composition of glycosaminoglycans in the Achilles tendon and thus may improve the mechanical properties of the Achilles tendon.

Key Words collagen peptide, tendon, collagen fibril, glycosaminoglycans, electronmicroscopy

Tendon is a major component of skeletal muscle tissues, and acts to transmit the force from skeletal muscle to bone. A major component of tendon is collagen, and the elastic characteristics of tendon depend on an ordered assembly of collagen fibrils (*1*). On the other hand, it has been reported that the oral ingestion of collagen peptide (CP) affects various tissues of the body. For example, CP ingestion enhanced bone mineral density of the mouse in protein undernutrition (*2*), and a joint disease was improved by daily ingestion of CP (*3*). It was also reported that the thickness of hair increased with prolonged ingestion of CP (*4*), and that a nail disorder such as brittle nails was improved by CP intake (*5*).

Although it is still unclear how CP is digested, is absorbed and subsequently affects various tissues, the synthesis of collagen is modulated by CP ingestion. Pacini et al. reported that the intravenous injection of CP enhanced the rate of collagen synthesis (*6*). Furthermore, CP promotes both procollagen type I biosynthesis and the repair process in a dermal wound (*7*). Therefore, it seems possible that the structure and function of tendon are also affected by the ingestion of CP because collagen is a major component of tendon. However, precisely how collagen fibrils in tendon respond to the ingestion of CP has yet to be thoroughly examined. In the present study, rabbits were fed with CP or with lactalbumin (LA) as a peptide control, and collagen fibrils of the Achilles tendon were examined by electron microscopy to determine how it was affected by the ingestion of CP or LA. The levels of another component of tendon, gycosaminoglycans (GAGs), were also determined biochemically.

MATERIALS AND METHODS

Rabbits and experimental design. All animal experiments in this study were approved by the Ethics Committee and the Institutional Animal Use and Committee of Sapporo Medical University prior to the experiments. Fifteen healthy adult male New Zealand White rabbits (Kbt:NZW, specific pathogen-free) weighing about 3.5 kg (3 rabbits/group) were purchased from Biotek Co. Ltd (Saga, Japan) and maintained in the animal facilities of Sapporo Medical University on a diet (CR-3, Clea Japan Inc., Tokyo, Japan) containing 17.2% protein (soybean meal). Daily intake of soybean meal was

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approximately 29 g/rabbit because a rabbit ingested about 175 g of the diet (50 g/kg body weight) per day. CP prepared enzymatically from swine skin was obtained from Nippi Inc. (PRA-PG; MW 3,000–5,000. Tokyo, Japan). Nitrogen content of CP was 15.7% as determined by a micro-Kjeldahl method, and CP was administered at 0.2 g/kg body weight (low dose; about 2% of the total protein intake) or 1.0 g/kg (high dose; about 12% of the total protein intake) per day. As a control protein to assess the effect of nitrogen intake, lactalbumin (LA) was used because LA dissolved readily in water. LA was administered at 0.23 g/kg (low dose) or 1.16 g/kg (high dose) so that the nitrogen intake per day was the same as that from CP because the nitrogen content of LA was 13.5%. Rabbits of the fifth group were given water alone. Both CP and LA were dissolved in 500 mL of water and fed in the morning. Water was fed ad libitum after the water bottle containing CP or LA became empty. All rabbits were killed on day 56 of the experiment and subjected to further examinations.

Transmission electron microscopy (TEM). The Achilles tendon of the central area in left hind limb was cut in blocks (1×1×3 mm) under a dissecting microscope and fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature. Samples were then washed in 0.1 M phosphate buffer and post-fixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer for 1 h at room temperature. Thereafter, the samples were washed with distilled water, dehydrated in a graded ethanol series, and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Sections approximately 60 nm thick were cut with a Reichert Supernova system (Leica, Austria) equipped with a diamond knife. The sections were mounted on a copper grid, stained with 1.0% uranyl acetate for 8 min, 1.0% lead citrate for an additional 10 min, and then examined by TEM (JEM-1220; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

For the measurement of collagen fibril parameters, 1,000 fibrils were randomly selected in photographs of each Achilles tendon. Then, the average collagen fibril diameter, mass average diameter (MAD), collagen fibril index (CFI), and number of collagen fibrils per μm^2 (density) of each rabbit were calculated. MAD is defined as the diameter of a fibril that contains the average mass present in the distribution and calculated as below where n_i is the number of measurements made for a fibril of diameter d_i and N is the number of diameter increments into which the fibril diameter distribution is divided (*8*):

$$MAD=\frac{\sum_{i=1}^{N} n_i d_i^3}{\sum_{i=1}^{N} n_i d_i^2}$$

MAD provides information on the tensile strength of a tissue that is not represented by a mean collagen fibril diameter (*9, 10*). CFI is the percentage of area covered by collagen and represents a collagen-to-noncollagen ratio in the extracellular matrix (*11*).

Analysis of glycosaminoglycans (GAGs). Each Achilles tendon was weighed and cut into small blocks at 4°C. After dehydration and degreasing by stirring in acetone 3 times for 30 min each at room temperature and then stirring in diethyl ether for 30 min 3 times at room temperature, the weight of the sample (dry weight) was determined. Removal of GAGs from core proteins of proteoglycans was performed by stirring samples in 0.5 N NaOH at 4°C for 15 h and then neutralizing it with 1 N HCl. Proteins in the mixture were denatured by heating at 100°C for 10 min. The mixture was brought to pH 8.0 with 1 M Tris-HCl buffer (pH 7.8) and digested with 1 mg/mL pronase (Actinase E; Seikagaku Kougyo, Tokyo, Japan) at 50°C for 24 h. After digestion with pronase, trichloroacetic acid was added to a final concentration of 10%. One hour later, the mixture was centrifuged at 1,600 ×*g* for 15 min to remove the precipitated proteins, and the supernatant was dialyzed against distilled water at 4°C for 4 d. The dialyzed sample was freeze-dried and subjected to two-dimensional electrophoresis on a cellulose acetate membrane. GAGs were stained with a solution containing 0.1% Alcian blue 8GX (Merck, Darmstadt, Germany) and 0.1% acetic acid. GAG content was quantified by assay for hexosamine according to the method of Hata (*12*). Hyaluronic acid (HA), dermatan sulphate (DS), heparin and chondroitin-6-sulphate (Nacalai Tesque, Kyoto, Japan) were used as standard GAGs.

Statistical analysis. A computer program (StatView for Windows, version 5.0) was used for the determination of means, standard errors, and one-way analysis of variance (ANOVA). Sheffé's test was used to compare differences among means of fibril diameter, MAD, CFI, density and the amount of GAG components at a significant level of $p=0.05$.

RESULTS

Histograms of collagen fibril diameter and TEM images of collagen fibrils are shown in Fig. 1. In the water control group, distribution of the diameter of collagen fibrils had a bimodal profile. Collagen fibril diameter was less than 340 nm, and fibrils of the diameter of 20–40 nm made up the highest percentage (Fig. 1e). In contrast, rabbits fed on CP or LA showed an altered distribution of fibril diameter; the number of collagen fibrils of larger diameter increased in comparison with the water control. It was also evident that fibrils of the diameter of 140–180 nm made up the highest percentage in the rabbits fed with the high dose of CP (Fig. 1b, arrow head) but not in the other groups. Fibrils with a diameter over 200 nm made up, however, the lowest percentage (8.3%) in rabbits fed with a high dose of CP; frequency of a diameter over 200 nm was highest (24.6%) for a low dose of CP (Fig. 1a, arrowhead), 19.3% for a low dose of LA, 14.8% for a high dose of LA, and 14.1% for the water control.

Mean diameter, MAD, CFI and density of collagen fibrils are summarized in Table 1. In rabbits fed on either the low or high dose of CP and with the low dose of LA, the average diameter of the collagen fibrils was significantly larger than that of the water control,

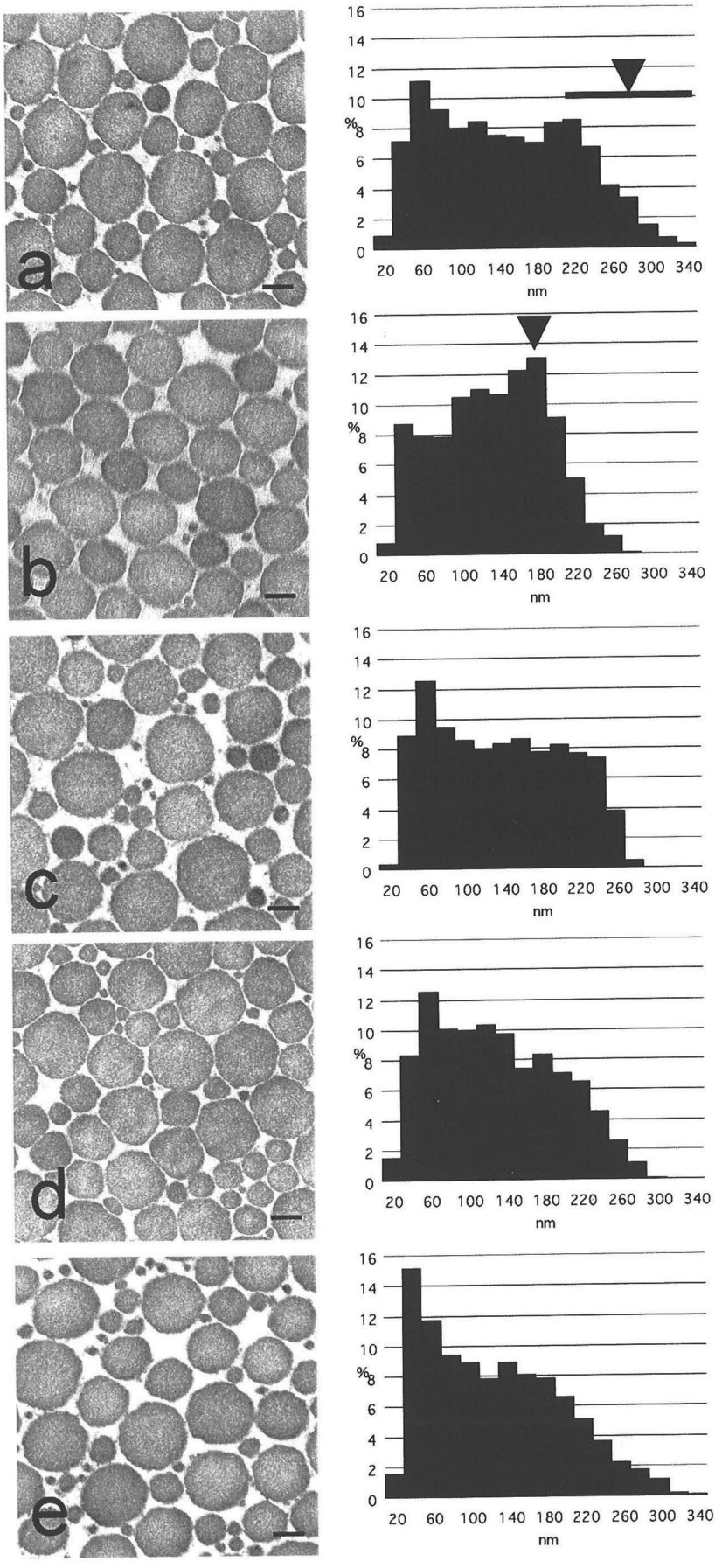

Fig. 1. TEM images and histograms of the diameter of collagen fibrils in the Achilles tendon. (a) low dose of CP, (b) high dose of CP, (c) low dose of LA, (d) high dose of LA, (e) water control. Arrowhead in (a) shows collagen fibrils larger than 200 nm, and arrowhead in (b) indicates the peak position. Bar=100 nm.

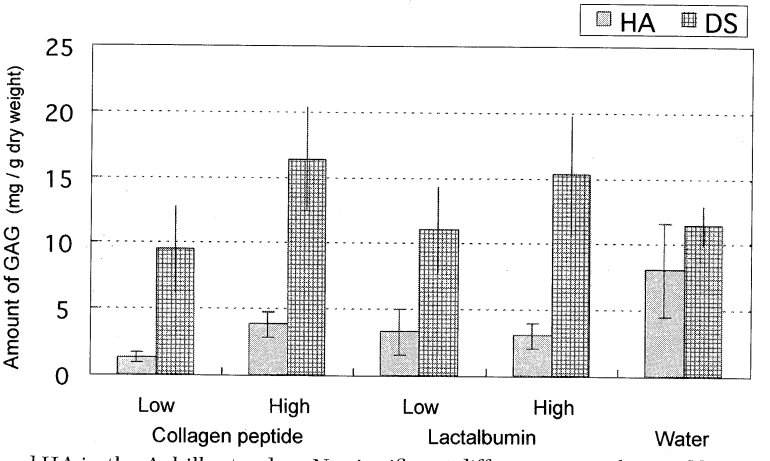

Fig. 2. Amount of DS and HA in the Achilles tendon. No significant difference was observed between experimental groups.

Table 1. Parameters of collagen fibrils and ratio of DS and HA in Achilles tendon.

Peptide dose	CP		LA		Water
	Low	High	Low	High	
Fibril diameter					
Mean±SE (nm)	131.2±1.4*	123.4±1.0*†	128.5±1.2*	121.2±1.2†‡	116.5±1.3
MAD±SE (nm)	201.4±8.0	170.7±8.3†	190.8±8.9	177.6±9.2	185.0±9.2
CFI±SE (%)	86.3±2.1	80.8±2.1	84.3±1.8	86.4±0.9	77.9±2.8
Density±SE (/μm^2)	37.3±2.7*	44.5±1.8*	41.4±2.0*	59.7±3.2†‡¶	62.2±4.6
Ratio of GAG components					
HA (%)	12.0	19.0	23.0	16.6	41.4
DS (%)	88.0	81.0	77.0	83.4	58.6

* Significantly different from water control.
† Significantly different from low dose of CP.
‡ Significantly different from low dose of LA.
¶ Significantly different from high dose of CP.

though there was no significant difference between rabbits fed on the high dose of LA or water. Fibril diameter in the high dose of LA was significantly smaller than those in the low dose of LA and the low dose of CP. In rabbits of the high-dose group of CP, MAD was significantly smaller than that of the low dose of CP. There was no significant difference in CFI among groups. In rabbits fed with either the high or low dose of CP and with the low dose of LA, fibril density was significantly lower than that in the water control. The density of the high dose of LA was significantly larger than those in the high and low dose of CP and the low dose of LA.

In the Achilles tendon, HA and DS were detected but heparin and chondroitin-6-sulphate were not found. The amount of HA and DS and their relative ratio are shown in Fig. 2 and Table 1, respectively. The amount of DS increased in rabbits fed with the high dose of CP or LA but was not statistically significant. In contrast, the amount of HA decreased when CP or LA was applied at both high and low doses in comparison with the water control (Fig. 2). Consequently, the relative amount of HA decreased markedly in rabbits fed with CP or LA (Table 1).

DISCUSSION

In the present study, the effects of ingestion of CP or LA on collagen fibrils and GAGs of the Achilles tendon of rabbits were examined, and it was found that the ingestion of CP and LA induced thickening of collagen fibrils and an altered composition of GAGs in the Achilles tendon.

The mechanical strength of tendon depends on the diameter of collagen fibrils as well as their organization; the collagen fibril of a smaller diameter is weaker than that of a larger diameter (*13*). In the present study, it was revealed that ingestion of CP or LA for 56 d resulted in an increase in the diameter of collagen fibrils. Although MAD did not increase significantly, these results suggest that the mechanical strength of collagen fibrils and consequently of the Achilles tendon may be enhanced by the ingestion of CP or LA.

It is worth mentioning that the ingestion of a high dose of CP resulted in a high percentage of collagen fibrils with a diameter of about 160–180 nm and a concomitant decrease in the percentage of fibrils with a diameter larger than 200 nm. This effect was not

observed in rabbits fed a high dose of LA nor rabbits that ingested a low dose of CP or LA. Therefore, it is suggested that the effect of CP on collagen fibrils in the Achilles tendon is different from that of LA and that the manner of effects of CP differs between low and high doses. The ingestion of collagen peptide at the dose of 0.2 g/kg may be most effective because the ratio of thick fibrils more than 200 nm in diameter was largest in this group, though we do not exclude the possibility that the high dose of collagen peptide, whose MAD was smaller than its low dose, is also beneficial because the ratio of moderately thick collagen fibrils was highest among the groups. Further studies should be carried out to elucidate the effect of CP ingestion on the mechanical properties of the Achilles tendon more precisely.

In the present study, a slight increase in the diameter of collagen fibrils in the Achilles tendon was observed in a rabbit administered a low dose of LA, and this effect was more evident in a high-dose group of LA. Digested and absorbed LA peptide might have a physiological activity to increase the diameter of collagen fibrils because the ratio of LA against ingested total protein was as small as about 2% in a low-dose group. On the other hand, the effects of CP and LA were different particularly at a high dose of ingestion. This implies that a mechanism that is specific to CP to control the size of collagen fibrils could exist in the Achilles tendon. Ingested protein is digested and usually appears as free amino acids in blood. Therefore, the differential effects of CP on collagen fibrils may be due to the particular amino acid composition of CP because CP contains about 33% glycine, and because a number of in vivo reactions are modulated by oral ingestion of glycine (*14, 15*).

However, it has also been reported that a considerable amount of CP-derived hydroxyproline appears in blood in a peptide form. Those hydroxyproline-containing peptides increased in amount after collagen intake and attained a peak level at 2 h after ingestion (*16, 17*). These CP-derived peptides in blood may affect functions of various tissues because, for example, tri-peptide Gly-Pro-Arg that could derive from CP shows anti-platelet effects in vitro and in vivo (*18*). It seems possible that the CP-derived peptides in blood induce the increase in the diameter of collagen fibrils observed in this study by enhancing collagen synthesis because CP reportedly promotes the synthesis of collagen (*6,7*).

It was reported that tissues with collagen fibrils of a larger diameter have high concentrations of DS (*19*). In the present study, the amount of DS was high in a rabbit fed with a high dose of CP or LA. These results suggest that the increase in fibril diameter may, at least partly, be due to the increased DS in the high-dose groups. It is worthwhile to note that the amount of HA decreased in rabbits fed with either a high or low dose of CP or LA. This suggests that HA may also play a role in controlling the diameter of collagen fibrils in the Achilles tendon because high concentrations of HA suppressed an increase in collagen fibril diameters (*14*). Mechanisms of the action of digested CP on tenocytes may reveal another aspect of the control of collagen fibrils in the Achilles tendon.

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