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Effects of Ingestion of Collagen Peptide on Collagen Fibrils and Glycosaminoglycans in the Dermis

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Summary In order to investigate the effects of collagen peptide ingestion on fibroblasts and the extracellular matrix in the dermis, collagen peptide was administered orally to pigs at 0.2 g/kg body weight/d for 62 d, and its effects were compared with those of lactalbumin and water controls. Fibroblast density, and diameter and density of collagen fibrils were significantly larger in the collagen peptide group than in the lactalbumin and water control groups. The two major components of dermal glycosaminoglycans, hyaluronic acid and dermatan sulfate, which are present in the inter-fibrillar space, did not differ significantly among the three groups. However, the ratio of dermatan sulfate, which is derived from fibril-bound decorin, was largest in the collagen peptide group. These results suggest that ingestion of collagen peptide induces increased fibroblast density and enhances formation of collagen fibrils in the dermis in a protein-specific manner.

Key Words collagen peptide, dermis, collagen fibril, glycosaminoglycan, electron microscopy

The skin is the largest organ in the human body and protects the body from various external insults. Skin comprises two layers, the epidermis and the dermis, consisting of stratified squamous epithelium and connective tissues, respectively. The dermis contains large amounts of extracellular matrix (ECM) components, such as collagen and glycosaminoglycans (GAG), mainly produced by fibroblasts (*1*). Collagen is the most abundant protein in the vertebrate body, comprising approximately 30% of the total protein. To date, over 20 types of collagen genes have been identified, and these are divided into three groups: fibrous collagen, fibril-associated collagen and basement membrane collagen (*2*). In the dermis, type I collagen forms collagen fibrils that are further organized into collagen fibers in association with other types of fibrous or fibril-associated collagen (*3*). The size of collagen fibrils is an important factor that determines the physical nature of tissue, as collagen fibrils/fibers form the framework of the vertebrate body (*4*). The size of collagen fibrils varies depending on tissue type and physiological conditions (*5*). Their diameters are reportedly regulated, for example, by the content of collagen types III or V or other types of non-fibrous collagen. Rates of synthesis and degradation of collagen also probably determine the size of collagen fibrils (*6*, *7*).

GAG consists of repeating two-sugar units and, with the exception of hyarulonic acid (HA), exists in tissue as proteoglycans that form covalent bonds with core proteins (*8*). The dermatan sulfate (DS) proteoglycan decorin is located on the surface of collagen fibrils and regulates their size (*9*). On the other hand, HA is highly hydrophilic and forms a gel with large amounts of water, playing a role in resisting external mechanical stresses (*8*, *10*). The change in the ratio of HA affects the diameter of the collagen fibrils (*11*). Thus, the size of collagen fibrils and the amount of GAG, such as DS (decorin) and HA, represent an important aspect of the mechanical properties of the dermis.

It has been reported that the oral ingestion of collagen peptides (CP) affects various functions of the body. For example, lower bone mineral density in protein malnutrition and joint disease are improved by ingestion of CP (*12*, *13*). It has also been reported that hair thickness increased after prolonged ingestion of CP (*14*) and that nail disorders, such as brittle nails, were improved by CP intake (*15*). Recently, we reported that ingestion of CP increases the size of collagen fibrils in the rabbit Achilles tendon in a dose-dependent and protein-specific manner (*16*). In the present study, the effects of oral ingestion of CP on fibroblast density (cell No. per unit area of a cross section of the dermis), size of collagen fibrils and GAG content in the dermis was investigated in order to examine whether CP ingestion affects the properties of ECM components in the dermis.

MATERIALS AND METHODS

Animals and experimental design. All animal experiments in this study were approved by the Ethics Com-

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mittee of Rakuno Gakuen University. A litter of 9 healthy 66-d-old piglets (Landrace, Large White stock crossbreeds) were divided into three groups (3 piglets/group) such that the average body weight did not differ among the groups (group 1: 41.7±14.2、group 2: 43.5±9.6、group 3: 45.7±3.1 kg, respectively), as no sex differences were observed in collagen fibrils in our previous study (*17*). Pigs were selected as experimental animals because their skin is used clinically as a skin substitute and because, like humans, they are omnivorous. Animal groups (3 animals per area) were fed a restricted diet of Superpork 80 (Chubu Shiryo Inc., Aichi, Japan) containing 15.0% protein (cereals). The control group (group 1) was fed a diet of Superpork 80. The CP group (group 2) was administered food containing CP (PRA-PG, MW=3,000–5,000, porcine skin-derived, Nippi Inc., Tokyo, Japan) such that a daily CP intake of 0.2 g/kg body weight was attained. In order to know whether the effect of CP ingestion is collagen-specific or is due to the ingestion of protein itself, the lactalbumin (LA) group (group 3) was administered food containing LA (D-500, Nippon Shinyaku Co. Ltd., Kyoto, Japan) at a dose of 0.23 g/kg body weight such that the nitrogen intake in the LA and CP groups was the same (nitrogen content of LA was 13.5%, while that of CP was 15.7%), as determined by the micro-Kjeldahl method. Body weight was measured every week. All animals were killed on day 62 of the experiment and subjected to further examination.

Light microscopy. Skin samples (20×20×20 mm) were obtained from the prescapula region of the neck skin and fixed in Bouin's fixative for 24 h at room temperature. After dehydration through ethanol and xylol series, samples were embedded in paraffin. Thin sections were cut at 6 μm, dewaxed and stained with Hematoxylin and Eosin. Dermal thickness was measured in 3 randomly selected sections of each animal under a microscope fitted with a micrometer at a magnification of 200. Nine values for each group were used for statistical evaluation.

In order to measure fibroblast density in the dermis, deparaffinized sections were stained by the avidin-biotin-peroxidase complex method (*18*) using rabbit IgG anti-prolyl 4-hydroxylase (beta) antibody (Daiiti Fine Chemical, Toyama, Japan).

Transmission electron microscopy (TEM). Skin samples (1×1×1 mm) were fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature. Samples were then post-fixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer for 1 h at room temperature. Thereafter, samples were washed with distilled water, dehydrated in a graded ethanol series, and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Sections of approximately 60 nm were cut with a Reichert Supernova system (Leica, Austria) equipped with a diamond knife. Sections were mounted on a copper grid and stained with 0.2% tannic acid+10% ethanol in water for 30 min. Sections were then stained with 1.0% uranyl acetate for 8 min and 1.0% lead citrate for an additional 10 min, and were then examined by TEM (JEM-1220; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

For measurement of collagen fibril parameters, 1,000 fibrils were randomly selected in photographs of each skin sample. The average collagen fibril diameter, collagen fibril index (CFI), and number of collagen fibrils per μm^2 (density) for each animal were calculated. CFI is defined as the percent area covered by collagen fibrils and represents the collagen to non-collagen ratio of ECM.

Analysis of GAG. Frozen skin samples were cut into small blocks on ice. After dehydration and degreasing by stirring in acetone 3 times for 30 min each at room temperature, and stirring in diethyl ether 3 times for 30 min each at room temperature, the weight of the sample (dry weight) was determined. Removal of GAG from proteoglycan core proteins was performed by stirring samples in 20 volumes of 0.5 N NaOH at 4°C for 15 h followed by neutralization 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. Trichloroacetic acid was then added at a final concentration of 10%. After 1-h incubation, the mixture was centrifuged at 1,600×*g* for 15 min in order to remove the precipitated proteins, and the supernatant was dialyzed against distilled water at 4°C for 3 d. The dialyzed sample was freeze-dried and subjected to two-dimensional electrophoresis on a cellulose acetate membrane. GAG 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 (*19*). HA, DS, heparin and chondroitin-6-sulphate (Nacalai Tesque, Kyoto, Japan) were used as GAG standards.

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

RESULTS

Morphological analysis

Throughout the experiment period, no significant differences were observed in body weight among the experimental groups. At the end of the experiment, the body weight of the control, LA, and CP groups was 108.2±27.4, 113.1±6.9, 105.4±11.2 kg, respectively. The effects of CP on dermal thickness, fibroblast density, diameter and density of collagen fibrils and the ratio of GAG are summarized in Table 1. No significant differences were observed in the thickness of the dermal layer among the groups. Prolyl 4-hydroxylase-positive fibroblasts (Fig. 1) were scattered in the dermal layer in all groups. In contrast to the thickness of the dermal layer, fibroblast density of the CP group was significantly

Table 1. Effects of CP ingestion on collagen fibril and GAG in the dermis.

Item	Control	LA	CP
Thickness of dermis (mm)	2.4±0.2	2.3±0.2	2.5±0.2
Density of fibroblast (cells/mm^2)	33.3±0.9	32.2±0.7	40.2±0.9*#
Diameter of collagen fibril (nm)	103.2±0.4	102.1±0.5	106.4±0.5*#
Density of collagen fibril (fibrils/μm^2)	77.9±2.7	74.3±2.2	90.5±1.8*#
CFI (%)	65.0±2.2	60.7±1.2	71.3±2.2*#
Ratio of GAG components			
HA (%)	52.9	50.4	48.3
DS (%)	47.1	49.6	51.7

Mean±SD.
*Significantly different from the control group ($p<0.05$).
#Significantly different from the LA group ($p<0.05$).

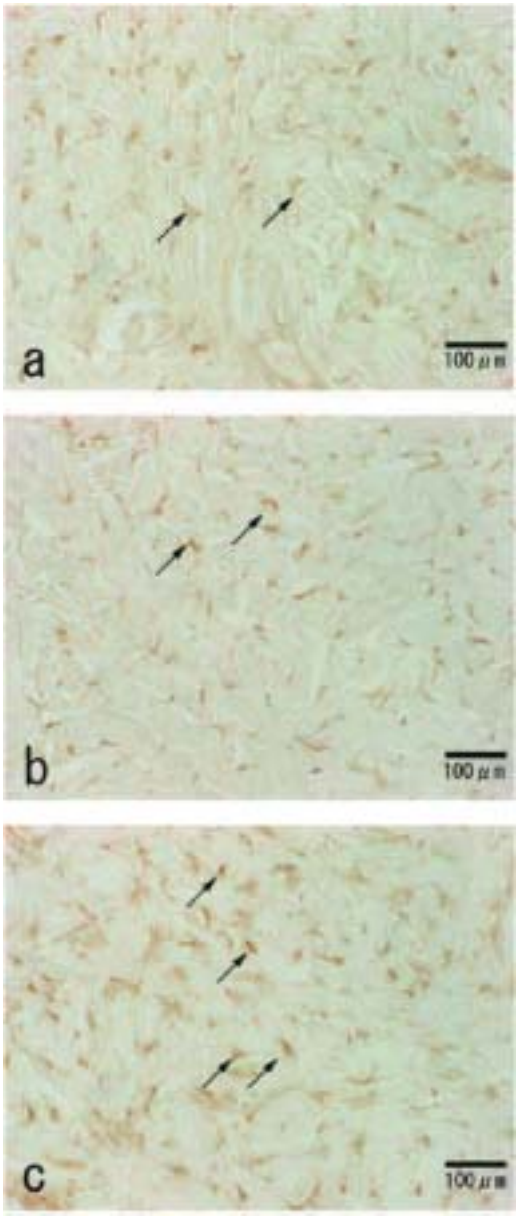

Fig. 1. Light micrographs of the dermis. (a) control, (b) LA, and (c) CP group. Dermal fibroblasts (arrows) were stained with rabbit IgG anti-prolyl 4-hydroxylase (beta) antibody. Bar=100 μm.

larger than that of the other two groups. Diameter and density of collagen fibril were also significantly higher in the CP group than the other two groups (Fig. 2). CFI value in the CP group was significantly higher than that in the other 2 groups, as both fibril diameter and fibril density were highest in the CP group.

Biochemical analysis

GAG in the dermis mainly comprised HA and DS, as reported previously (*17*). Although the ratio of DS was higher in the CP group, no significant differences were observed between each pair of groups.

DISCUSSION

The effects of oral ingestion of CP on dermal fibroblasts and ECM components, i.e., collagen fibrils, DS and HA, were examined in pigs, and it was found that CP ingestion induced significant increases in the diameter and density of collagen fibrils, as well as the density of fibroblasts. It is noteworthy that these effects were observed in pigs fed with normal food containing collagen peptide.

Collagen fibrils are polymerized into collagen fibers to form a backbone structure of connective tissue. It has been reported that collagen fibrils with smaller diameters are mechanically weaker than thicker fibrils (*20*). In the present study, ingestion of CP induced a significant increase in both diameter and density of collagen fibrils. Consequently, CFI was highest in the CP group. These results suggest that prolonged ingestion of CP improves the mechanical properties of the dermis to resist external mechanical insults by enhancing the formation of collagen fibrils. It seems likely that this effect is achieved, at least partly, by the proliferation of fibroblasts, as their density also increased significantly (*21*). It is worth noting that the control protein (LA) showed no such effect. This implies that the effect of CP was protein-specific and did not depend simply on increased amino acid intake. We have also reported that the ingestion of CP resulted in a significant increase in the diameter and density of collagen fibrils in rabbit Achilles tendon in a dose-dependent and protein-specific manner (*16*). These studies suggest that ingestion of CP has specific effects on collagen fibrils in collagen-rich

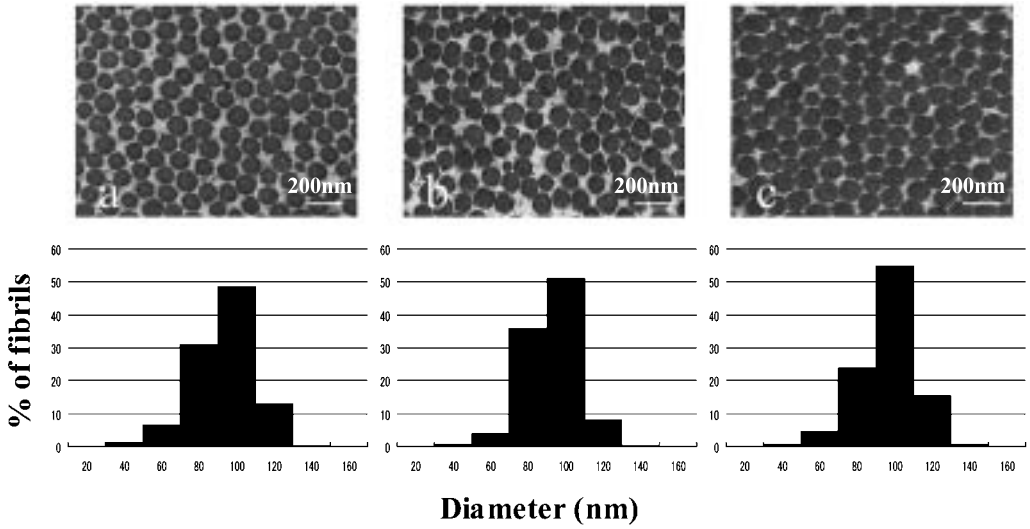

Fig. 2. Transmission electron micrographs of collagen fibrils in the dermis and histograms of collagen fibril diameter. (a) control, (b) LA, and (c) CP group. Bar=200 nm.

tissues, such as the Achilles tendon and the dermis. Iwai et al. (*22*) recently reported that the ingestion of collagen peptide results in the appearance of collagen-derived oligo-peptides in serum. It thus seems possible that these collagen-derived oligo-peptides affect the proliferation of fibroblasts and the formation of collagen fibrils in a collagen-specific manner.

GAG in the skin mainly comprises HA and DS (*17*). This was confirmed in the present study in all 3 experimental groups. HA forms a gel with large amounts of water and possesses high swelling pressure (*8*, *10*). Thus, it is assumed that HA plays an important role in resisting external pressure (*11*). Conversely, DS exists on the surface of collagen fibrils in the form of the DS proteoglycan decorin. Decorin transmits force to other collagen fibrils by interconnecting among collagen fibrils (*23*). Decorin also functions to resist compression, to regulate the diameter of collagen fibrils, and to facilitate fibril elongation (*8*, *24*, *25*). The amount of HA and DS varied based on ingestion of LA or CP, and the ratio of DS was highest in the CP-fed group. This agrees with the fact that the amount of collagen fibrils, as indicated by CFI, was also highest in this group.

We previously reported that the water absorption potential of the stratum corneum in adult women was increased by daily ingestion of 10 g CP for 60 d (*26*). This implies that ingestion of CP improves the function of the outermost part of the epidermis where no collagen fibrils or fibroblasts are found. Similar effects of gelatin ingestion on cutaneous hydration were reported by Morganti et al. (*27*). Taken together with these studies, the present study suggests that the ingestion of CP improves the function of both the epidermis and the dermis.

In summary, the present study suggests that ingestion of CP induces increases in diameter and density of collagen fibrils in the dermis. It is thus possible that ingestion of CP improves the mechanical strength of the skin and prevents skin injury under pathologic and/or normal conditions. However, further examination is necessary to confirm this notion.

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