FULL PAPER *Anatomy*

Effect of Collagen Oligopeptide Injection on Rabbit Tenositis

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ABSTRACT. Effects of the collagen oligopeptide (COP) were examined by its repeat injection into the inflammatory rabbit Achilles tendon (*Tendo calcaneus communis*), in which tenositis was induced by injection of bacterial collagenase. COP was evaluated 5 times over a period of 3 weeks to 1 month after injection of collagenase. At 1 month after treatment, the therapeutic effect of COP was evaluated by examining the structure of collagen fibrils, amount and components of glycosaminoglycans (GAGs) and matrix metalloproteinases (MMPs), and compared with the saline injection, control, and normal groups. The Achilles tendon of rabbit in the control group (no COP injection) and saline injection group showed a notable increase in the number of fine collagen fibrils, a change in GAG composition and increase in the amount of pro-MMP-2, indicating the weakening of the tendon. In contrast, the size distribution of collagen fibrils, GAG composition and the amount of pro-MMP-2 was similar to those in the normal group. These results suggest that COP injection promotes healing processes of the tendon injury.

KEY WORDS: collagen fibril, collagen oligopeptide, electron microscopy, glycosaminoglycans, rabbit tendon.

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The basic function of a tendon is to convey the force exerted by a muscle to the bone. The mechanical load is not transmitted directly to the bone because tendon is a highly flexible connective tissue. This connective tissue is composed of two components: tendon cells and extracellular matrix (ECM) that consists of collagen and proteoglycans [1, 21]. Collagen in a tendon is composed of mainly type I collagen and is arranged into bundles of collagen fibrils that are further organized into tendon fascicles [22]. Proteoglycan has its core protein covalently bound to glycosaminoglycan (GAG). GAG is a non-branched polysaccharide consisting of repeated units of amino acids and uronic acids. GAGs in ECM exist as proteoglycans except for hyaluronic acid (HA) that exists as a free GAG. Proteoglycans are classified into chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS), heparin or keratan sulphate according to their uronic acid subunit [3]. Decorin is the most abundant DS proteoglycan in tendon, and is reportedly involved in both the formation of collagen fibrils and adjustment of collagen fibril diameter [37]. However, its role in the turnover of tendon ECM has not yet been elucidated [16].

Matrix metalloproteinase (MMP) is an enzyme that has zinc ion in its active core and requires calcium ion for maintenance of the activity. More than 20 types of MMP have been reported so far, and they play an important role in the turnover of tendon ECM [7, 34]. MMPs are divided into the following types: collagenase (MMP-1, -8, -13), gelatinase (MMP-2, -9), stromelysin (MMP-3, -10), membrane (MMP-14, -15, -16, -17, -24, -25), and others (including MMP-7, -11, -12) according to their biochemical properties [4]. However, how these MMPs are involved in the turnover of tendon ECM remains unclear.

Inflammation of a tendon or tendon-muscle junction is a serious problem in both humans and animals, particularly racehorses [20]. Tenositis in racehorses results in the reduced performance which indicates a significant economic loss [16]. Complete cure of the tenositis is difficult, and the recurrence of the tenositis that accompanies scar formation is observed frequently [26, 33]. Tendon injury could be caused sudden load or accumulated fatigue that induces the degeneration of ECM through the imbalance of the turnover of ECM components [5, 16, 29, 33]. A number of mechanisms of the tenositis have been suggested, e.g., 1) a physical mechanism by which repetitive physical forces acting directly on the tendon cause accumulation of damage to the ECM structure, 2) a physiological mechanism by which ECM degeneration is caused by a rise in temperature in the tendon due to heat generated by exercise, 3) a vascular mechanism by which tendon cells and ECM are destroyed by hypoxia and free radicals that are induced by stoppage and subsequent resume of the blood flow in tendon, respectively, and 4) a proteinase mechanism by which ECM degradation is caused by the synthesis, secretion and activation of proteinases due to various stimuli [31].

Various methods for treating the tendon injury have been tested in clinical trials [9]. But their results are inconsistent, and there is skepticism regarding their effectiveness. Therefore, development of a new effective therapeutic method important subject including, for example, suppression of the degenerative changes in ECM prior to the onset of clinical tenositis, or the development of a method for rapid regener-

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ation of the near-normal tissue instead of scar formation. To establish a new therapeutic method, both *in vivo* studies using horses and other experimental animals and *in vitro* studies using cultured cells have been carried out [7, 13, 33, 35]. Among those trials, a tendon injury model using rabbits is a cheaper and easier system to handle than using horses [8, 32, 38, 39]. In this rabbit model, bacterial collagenase was injected into a tendon to induce tenositis whose histology is the same as that of naturally occurring tenositis [8, 32, 39].

Although mechanisms of tenositis are still unclear, an previous *in vivo* study showed that the administration of collagen peptide results in an increase in the ratio of collagen synthesis [24]. Therefore, in this study, tenositis was produced experimentally in rabbits using collagenase, and the therapeutic effects of collagen oligopeptide (COP) injection into the tendon were investigated morphologically and biochemically.

MATERIALS AND METHODS

Collagen peptide (COP): Collagen prepared from the embryonic bovine skin by pepsin treatment [10] was digested with bacterial collagenase (Sigma-Aldrich, St.Louis, MO, U.S.A.) at 37°C for 5 hr. Digested collagen peptide in 50 mM NaCl was passed through Superdex peptide HR 10/30 (GE Healthcare UK Ltd, England) at 0.5 m*l*/min under the monitoring at 220 nm, and fractions of retention time 29–35 min was isolated. The fractions were lyophilized and redissolved in water at 0.3 mg/m*l*.

Rabbits and the experimental design: Twelve healthy adult male New Zealand White rabbits weighing about 3.5 kg (3 rabbits/group) were obtained from Japan SLC (Hamamatsu, Japan) and maintained in the animal facilities of Sapporo Medical University. The Animal Experimental Ethics Committee in Sapporo Medical University approved the studies. Ten days after the induction of tendon injury by injection of 1,200 IU collagenase (Type-1-S; Sigma-Aldrich, St Louis, MO, U.S.A.) into the Achilles tendon (*Tendo calcaneus communis*) of both hind legs of 9 rabbits, 3 rabbits received the injection of COP into the tendon of the right hind legs (the treatment group; see below for details about the treatments of injury). The injection parts were center of the Achilles tendon of 10 mm up from mass calcaneus. The other 3 rabbits were injected with the same volume of physiological saline into the right hind legs (the saline injection group), and both legs of the other 3 rabbits were untreated (the control group). The left hind legs of these 9 rabbits were untreated after the injection of collagenase. The remaining 3 rabbits (the normal group) did not undergo tendon injury. Rabbits were sacrificed 2 months after treatment, and the Achilles tendon taken *post mortem* from both hind legs was each divided into two portions for ultrastructural examination and GAG analysis.

Treatment of injury: Treatment was based on the method of Spurlok *et al.* [33] and Reef *et al.* [27]. Before the injection of COP or saline, local anaesthesia was induced with lidocaine hydrochloride. Three hundred μl COP in physiological saline or physiological saline alone was then injected into the medial core region of the injured tendons. Treatment was performed five times at the intervals of 3 days starting on day 21.

Transmission electron microscopy (TEM): The center area of the Achilles tendon of the left hind limb was dissected 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 hr 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 hr 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, mounted on a copper grid, then stained with 1.0% uranyl acetate for 8 min and 1.0 % lead citrate for an additional 10 min before being 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 selected randomly in photographs of each Achilles tendon. The average collagen fibril diameter, mass average diameter (MAD), collagen fibril index (CFI), and the number of collagen fibrils per μm^2 (density) of each rabbit were then calculated. MAD was defined as the diameter of a fibril representative of the average mass within the overall distribution and calculated as follows [12]:

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

where n_i is the number of measurements made of a fibril of diameter d_i and N is the number of diameter increments into which the fibril diameter distribution is divided. MAD provides the information on the tensile strength of a tissue not represented by a mean collagen fibril diameter [11, 19]. CFI is the percentage of area covered by collagen and represents the collagen-to-noncollagen ratio in the ECM [6].

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 each at room temperature, the weight of the samples (dry weight) was determined. Removal of GAGs from core proteins of the proteoglycans was performed by stirring samples in 0.5 N NaOH at 4°C for 15 hr and then neutralizing them with 1 N HCl. Proteins in the mixture were denatured by heating at 100°C for 10 min. The mixture was then brought to pH 8.0 with 1 M Tris-HCl buffer (pH 7.8) and digested with 1 mg/m*l* pronase (Actinase E; Seikagaku Corporation, Tokyo, Japan) at 50°C for 24 hr. 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 days. The dialyzed samples were freeze-dried and then 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 hexosamine assay according to the method of Hata [17]. HA, DS, heparin and chondroitin-6-sulphate (Nacalai Tesque, Kyoto, Japan) were used as standard GAGs.

Analysis of MMP: Gelatin, casein and collagen zymography were conducted to determine the activity of collagenase (MMP-1), gelatinases (MMP-2 and -9) and stromelysin (MMP-3) using 8% gels containing 1 mg/m*l* gelatin and 10% gels containing 1 mg/m*l* β-casein or type I collagen. Each band area was measured by densitometric analysis (NIH Image). Protein marker (BIO-RAD Laboratories, CA, U.S.A.) were used as identification of MMPs.

Statistical analysis: 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, and Student's *t* test was used to compare differences in MMP compartments at a significance level of P=0.05.

RESULTS

Tendon samples obtained from the treatment, control and saline injection groups were more yellow in color, more swollen and softer than samples from the normal group. The results of morphological analyses are shown in Fig. 1 and Table 1. The diameter of collagen fibrils in the normal group was between 10–340 nm with two peaks at 20–40 nm and 240–260 nm. Samples from the control group had many fine fibrils ranging from 10–260 nm with one peak at 60–80 nm, and those in the saline injection group distributed from 10–280 nm with one peak at 20–40 nm. The diameter of fibrils in the treatment group was between 10–310 nm with two peaks at 20–40 nm and 160–180 nm (Fig. 1). The MAD in the normal group was 193 ± 7.1 nm, which was higher than that in the control group and the saline injection groups (153 ± 7.8 nm and 183 ± 7.4 nm, respectively). In contrast, MAD of the treatment group (194 ± 6.8 nm) was almost the same as that of the normal group (193 ± 7.1 nm). The density of collagen fibrils in the normal group was 33.4 ± 3.1/μm^2, which did not differ significantly from that in the treatment group (32.5 ± 4.3/μm^2). In contrast, the density was significantly higher in the control and saline injection groups (64.1 ± 18.1/μm^2 and 85.1 ± 32.4/μm^2, respectively) (Table 1).

GAGs in the tendon were composed of two components HA and DS in all groups. The amounts of HA and DS were 3.0 ± 0.08 mg/DW and 26.5 ± 11.8 mg/DW, respectively, in the normal group, 0.7 ± 0.08 mg/DW and 5.6 ± 0.8 mg/DW in the control group, 2.7 ± 1.3 mg/DW and 22.5 ± 5.0 mg/DW in the treatment group, and 1.3 ± 0.2 mg/DW and 7.3 ± 1.3 mg/DW in the saline injection group (Table 1). There were no significant differences between each groups in the amounts of GAGs.

The activity of pro- and active MMP-2 were detected, the amounts being respectively 0.04 ± 0.01 μg and 0.02 ± 0.007 μg in the normal group, 0.2 ± 0.2 μg and 0.042 ± 0.01 μg in the control group, 0.01 ± 0.007 μg and 0.037 ± 0.02 μg in the treatment group, and 0.02 ± 0.005 μg and 0.07 ± 0.02 μg in the saline injection group (Table 1). There were no significant differences between each group in the amount of MMP.

DISCUSSION

Collagen fibrils are basic units that provide tendons with tensile strength, and the mechanical properties of the tendon are reportedly correlated with the distribution of collagen fibril diameter [23]. Collagen fibrils in a mature tendon can be divided into fine fibrils (40 nm in diameter), medium-sized fibrils (120 nm in diameter) and thick fibrils (> 200 nm in diameter) [1, 25, 30]. Fine fibrils fill in the gaps between thick fibrils, preventing degeneration of the tendon when tensile force is applied, while thick fibrils have a high density of cross-linking between molecules, providing tensile strength. Thus, both fine and thick collagen fibrils are necessary for tendon strength [23, 25, 26, 30].

In the present study, the distributions of collagen fibrils in the control group and saline injection group showed one peak, whereas those in the normal group and treatment group showed two peaks. This difference in distribution was probably due to an increase in the number of fine fibrils in the control and the saline injection groups. These results suggested that tenositis was associated with the increase in the number of fine collagen fibrils, and that the injection of physiological saline did not improve the inflammatory status of the tendon. It was also suggested that the injection of COP had the clear therapeutic effects on the tenositis induced by the injection of collagenase.

The MAD of the treatment group was similar to that of the normal group, and MAD of these two groups was significantly higher than those of the control group and the saline injection groups. These results were consistent with the fact that fine collagen fibrils were less frequent in the normal group and the treatment group, and suggested that the strength of the tendon was improved by the injection of COP because fine fibrils had less resistance to elongation [16].

Biochemical analysis in the present study showed that the GAGs in the tendon consisted of DS and HA. DS was the main GAG in the normal rabbit tendon, though there are reports of HA and CS also being detected [14] and of CS not being detected [28] in the normal tendon. The amounts of DS in the control group and the saline injection group were less than in the normal group. On the other hand, the amount of DS in the treatment group was similar to that in the normal group and there was no change in GAG composition, suggesting that the injection of COP showed therapeutic effects also on the composition of GAGs.

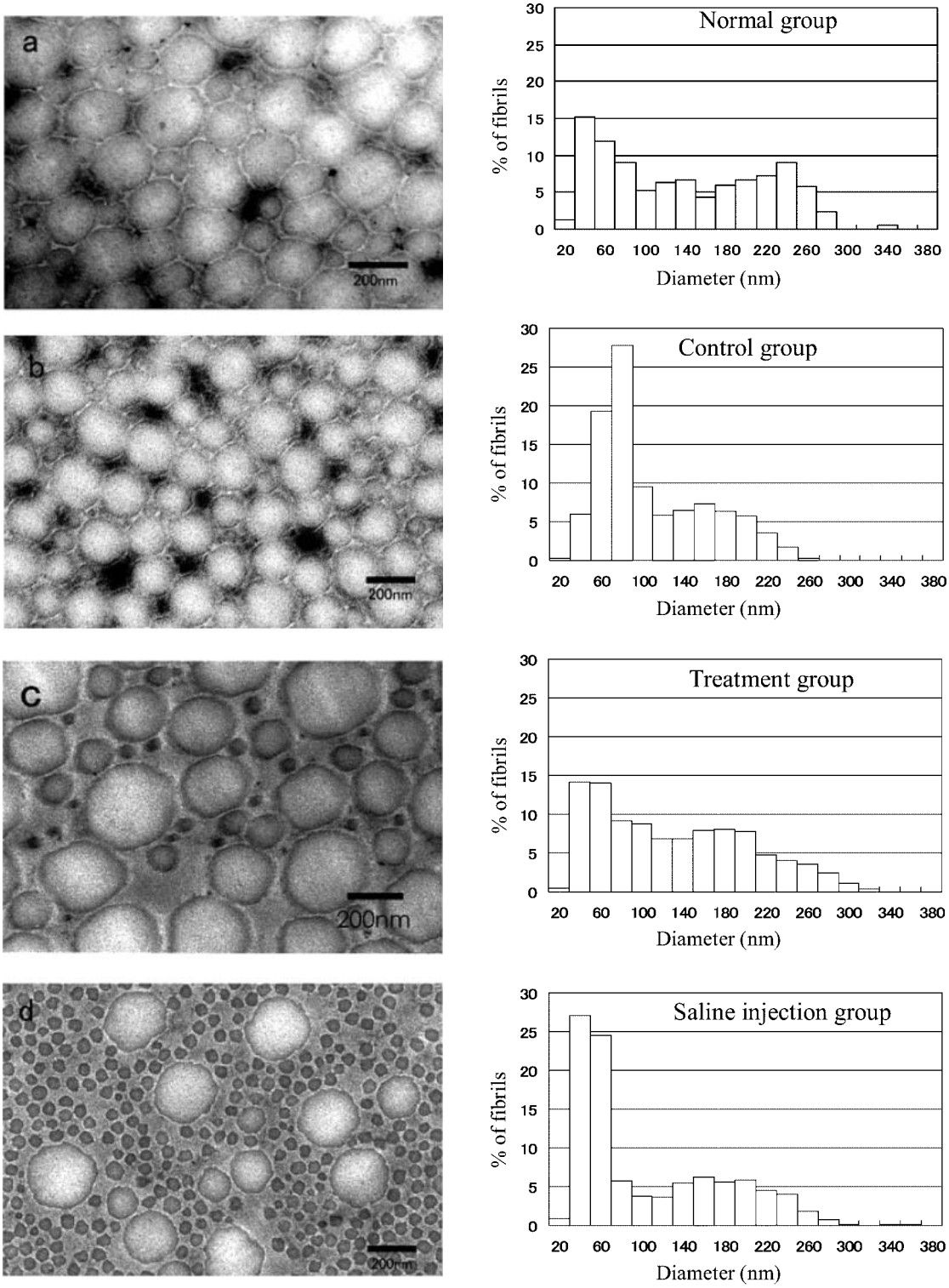

Fig. 1. TEM images and histograms of the diameter of collagen fibrils in the Achilles tendon of (a) the normal group, (b) the control group, (c) the treatment group, and (d) the saline injection group. Bar=200 nm.

Table 1. Parameters of Collagen fibril, ratio of DS and HA, and components of MMP

	Normal group	Control group	Treatment group	Saline injection group
Collagen fibrils				
Diameter (nm)	10–340	10–260	10–310	10–280
MAD ± SE (nm)	193 ± 7.1	153 ± 7.8	194 ± 6.8	183 ± 7.4
Density ± SE (/μm^2)	33.4 ± 3.1	64.1 ± 18.1*	32.5±4.3	85.5 ± 32.4*
Ratio of GAG component				
HA ± SE (mg/DW)	3.0 ± 0.08	0.7 ± 0.08	2.7 ± 1.30	1.3 ± 0.20
DS ± SE(mg/DW)	26.5 ± 11.8	5.6 ± 0.80	22.5 ± 5.00	7.3 ± 1.30
Components of MMP				
Pro-MMP-2 (μg)	0.04 ± 0.010	0.20 ± 0.200	0.01 ± 0.007	0.02 ± 0.005
Active- MMP-2 (μg)	0.02 ± 0.007	0.04 ± 0.010	0.04 ± 0.020	0.07 ± 0.020

MAD: mass average diameter, GAG: Glycosaminoglycan, HA: Hyaluronic acid, DS: Dermatan sulfate, DW: dry weight.
* Significantly different from normal group.

The notable increase in the amount of pro-MMP-2 in the control group indicated that the healing process of the injured tendon was associated with the increase in the amount of pro-MMP-2. It was also evident that the COP treatment promoted the healing process because pro-MMP-2 decreased in amount in the treatment group. It was noteworthy that the amount of pro-MMP-2 was small in the saline injection group in which no therapeutic effects were observed with regard to collagen fibril diameter, MAD and density. Although the reason for the small amount of pro-MMP-2 in the saline injection group was not clear, it may be possible that pro-MMP-2 was diluted or degenerated by edema of the Achilles tendon in the saline injection group in which the number of small fibrils increased markedly.

The mechanism by which COP injection promoted healing processes after injury of the Achilles tendon was not clear at present. However, it was unlikely that COP acted as an inhibitor of injected collagenase since COP was infused 21 days after induction of inflammation by collagenase. Since it was reported that collagen oligopeptides activated fibroblasts [24], the repeated injections of COP might act on cells producing collagen, GAGs and MMP-2 and affect the thickness of the collagen fibrils and the amounts of GAGs and pro-MMP-2. Although the turnover of collagen was not measured in the present study, degradation of collagen fibrils by MMP-2 might be inhibited because the amount of pro-MMP-2 decreased in the treatment group and pro-MMP-2 degrades not only degenerated collagen but also type I collagen [2]. It was suggested that degradation of collagen fibrils by pro-MMP-2 was inhibited.

DS in the Achilles tendon is a component of the proteoglycan decorin, the core protein of which is reportedly degraded by MMP-2 [18]. Since there is thought to be a correlation between the amount of decorin and the thickness of collagen fibrils [29], it is possible that suppression of an increase in pro-MMP-2 led to suppression of the degradation of decorin, contributing to the maintenance of collagen fibril thickness and thus promoting tendon repair.

It has been reported that increased turnover due to the progression of ECM degeneration by MMPs results in weakening of collagen fibrils in the tendon leading to clinical inflammation [30, 36], and subsequent formation of weak scar tissue leads to chronic tendon injury [15, 30]. Taken together, the present study suggests that COP injection promotes healing of the tendon injury, and that the injection of COP could be a novel therapeutic tool for tendonitis.

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