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Effects of oral administration of tripeptides derived from type I collagen (collagen tripeptide) on atherosclerosis development in hypercholesterolemic rabbits

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Digestion of type I collagen with a collagenase-type protease yields a collagen tripeptide (Ctp) fraction comprising Gly-X-Y sequences that exhibit diverse biological activities. We previously demonstrated that Ctp inhibits the proliferation and migration of cultured aortic smooth muscle cells (SMCs) *in vitro*. These cells contribute to the pathogenesis of atherosclerosis and other cardiovascular diseases. In order to evaluate the effects of Ctp on atherosclerosis development *in vivo*, here we used the Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbit model of familial hypercholesterolemia to determine the effects of oral administration of Ctp for three months. Ctp induced a significant decrease in the area occupied by atherosclerotic plaques in the aorta and in the level of total serum cholesterol. The components of atherosclerotic plaques underwent distinct changes, including reduction in the populations of macrophages and SMCs and a significant decrease in the proportion of macrophages to SMCs. Ctp administration decreased the number of cells in plaques that expressed proliferating cell nuclear antigen and the number of cells with oxidative damage to DNA as indicated by 8-hydroxy-2′-deoxyguanine detection. These findings are the first to define the mechanism underlying the inhibitory effects of Ctp on atherosclerosis development in hypercholesterolemic rabbits, and suggest that Ctp provides an effective therapy for treating atherosclerosis.

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[**Key words:** Collagen tripeptide; Atherosclerosis; Plaque; Kurosawa and Kusanagi-hypercholesterolemic rabbit; Smooth muscle cells; Total cholesterol]

Atherosclerosis is the principal cause of most diseases related to cardiovascular disease-related deaths in both the developed and developing countries (1). Long considered to arise from the progressive accumulation of lipids within the arterial wall, atherosclerosis is now recognized as a chronic inflammatory disease (2–4). Although effective measures to prevent and treat atherosclerosis are available, it remains refractory. Smooth muscle cells (SMCs) and macrophages derived from tissues and blood, respectively, are the most important cells in atherosclerotic lesions because they play crucial roles in determining disease development and prognosis (5,6). Moreover, any disturbance of their homeostasis may directly decrease the stability of the atherosclerotic plaque and cause thrombotic complications (7).

Macrophages internalize atherogenic lipoproteins such as oxidized low-density lipoproteins (LDLs) via scavenger receptors, and the generation of lipid-loaded macrophages containing large amounts of cholesterol esters (foam cells) is a hallmark of early and late atherosclerosis (4,8). The ability of macrophages to produce cytokines such as tumor necrosis factor-α, interleukin-1, and transforming growth factor-β; proteolytic enzymes, particularly matrix metalloproteinases (MMPs); and growth factors such as platelet-derived growth factor and insulin-like growth factor I is critical to their role, along with SMCs, in the damage and repair that ensue as atherosclerotic lesions progress (4). SMCs in the lesions synthesize the bulk of the connective tissue matrix, including collagen, elastic fibers, and proteoglycans (9,10). Thus, SMCs play the principal role in the fibro-proliferative component of this disease. The proliferation of macrophages and SMCs is therefore an important feature of atherosclerosis.

Using a protease that specifically hydrolyzes the peptide bonds of type I collagen at the amino terminus of glycine residues, we obtained a highly purified, nonantigenic, and low-allergenic tripeptide fraction containing Gly-X-Y sequences (where X and Y represent any amino acid residue, but most frequently proline and hydroxyproline) called collagen tripeptide (Ctp) (11). After oral administration, evidence indicates that intact Ctp is transported to the blood from the small intestine, for example, the transport of tritium-labeled Ctp (Gly-Pro-Hypro) was rapid in comparison with the absorption of tritium-labeled proline (12).

Ctp exerts multiple effects on cultured cells as well as on experimental animals and affects the function of many organs and tissues, including skin, bone, and cartilage (12–14). For example, Ctp apparently accelerates the healing of bone fractures (12), stimulates the calcification of human osteoblastic cells (13), and increases the production of type I collagen by human osteoblastic

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cells (13). DNA microarray and quantitative RT-PCR analyses demonstrate that Ctp up-regulates osterix, which is a bone-specific transcription factor (13). Moreover, Ctp enhances the production of hyaluronic acid and collagen by human dermal fibroblasts *in vitro* and in murine skin *in vivo* (15). Oral administration of Ctp in a mouse model of dry skin significantly reportedly decreased the transepidermal loss of water, suppressed scratching behavior, and dramatically inhibited the growth of intraepidermal nerves. Quantitative PCR and immunohistochemical analyses reveal that the increased levels of nerve growth factor and Sema3A returned to normal in a mouse model of dry skin and rehydrated the epidermis (15).

In our previous study on atherosclerosis, we found that Ctp inhibits the proliferation and migration of cultured human aortic SMCs and increases the synthesis of type I and IV collagen *in vitro* (16). The obtained results provided a clue for understanding the mechanism underlying the modulatory effects of Ctp on the pathogenesis of atherosclerosis.

The Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbit is a useful model for studying familial hypercholesterolemia (17–19) caused by the deficiency of the LDL-receptor, which leads to persistent hypercholesterolemia and the gradual development of atherosclerosis (20). Here we determined the effects of Ctp on atherosclerosis development using this rabbit model.

MATERIALS AND METHODS

Animal model Ten male KHC rabbits aged 3 months and weighing 2.275 ± 0.167 kg mean ± standard deviation (SD) were purchased from Japan Laboratory Animals (Tokyo, Japan) and were randomly separated into a control and an experimental group (hereinafter, Ctp group), comprising five rabbits each. The rabbits in the Ctp group were continuously administered Ctp (400 mg in 5 ml of distilled water) daily via a gastrointestinal feeding tube for three continuous months. The rabbits in the control group received 5 ml of distilled water using the same route. All rabbits were housed individually in a pathogen-free animal facility under a 12 h-dark/12 h-light cycle with free access to food and water. All protocols in this study were performed in accordance with the guidelines of the Animal Research Committee of Kanazawa Medical University.

Ctp preparation Ctp was prepared as previously described (9). Briefly, Ctp was prepared from porcine skin collagen digested using a collagenase-type protease (Protease N, Nagase Chemtex Corporation, Osaka, Japan). The digest was deionized using an ion exchange resin (Diaion type SK, Mitsubishi Chemical, Tokyo, Japan), passed through a 0.2-μm filter, and was then subjected to ion exchange chromatography using Toyopearl DEAE-650 (Tosoh Corp., Japan). The tripeptide fraction was isolated using reverse-phase high-pressure liquid chromatography (HPLC). The mean molecular mass of Ctp is approximately 1970 Da. The CTP contains more than 25% of the tripeptide component having the sequence (Gly-XY). The remaining 75% is composed of dimers/trimers of (Gly-X-Y) which means the precursor of tripeptides (10). Its major components of tripeptide include peptides such as Gly-Pro-Hypro (9.7%), Gly-Pro-Ala (4.7%), Gly-Ala-Hypro (1.7%), and Gly-Pro-Pro (1.1%). The purity of Ctp was analyzed using HPLC with a Superdex Peptide gel filtration column (GE Healthcare UK Ltd., UK) and quantitated by integrating the absorbance at 214 nm of the major peak. The composition of tripeptide components is expressed as the area ratio (%).

Analysis of serum lipids Blood samples were collected from an aural artery and added to tubes containing an anticoagulant. Total serum cholesterol (TC), HDL-cholesterol (HDL-C), and total triglyceride (TG) contents were determined using a cholesterol (Determiner-L TC II), triglyceride (Determiner-L TG II) (Kyowa Medex, Tokyo, Japan), and cholesterol Qualigent HDL (Sekisui Medical, Tokyo, Japan) kit, respectively.

Quantitative analysis of atherosclerotic plaque area in the aorta Rabbits were sacrificed using an injection of an overdose of sodium pentobarbital. A thoracotomy and laparotomy were performed to expose the heart and the entire aortal tree. The aorta was separated and removed intact from its origin to the origin of the iliac arteries. After the aorta was cut along its length and fixed with 4% paraformaldehyde, photographs were taken at the same magnification. The aortic lumen involved in the lesion was selected manually according to the different color by the computer-assisted image analysis software Image-Pro Plus 5.1. Briefly, by using the menu of "select color" and "color cube based", the parts of lesion were chosen and shown in red color by the software automatically (shown in Fig. 2). Then the percentage of lesion was calculated by the software and the statistic analysis was performed.

Immunohistochemistry The aortas were cut in cross-section into small segments (5-mm each), embedded in paraffin, and stained with hematoxylin–eosin and Elastica van Gieson. Representative lesioned segments were selected, and immunohistochemical analysis was performed using specific antibodies and the streptavidin-biotin immunoperoxidase procedure. For the double-labeling procedure, sequential avidin-biotin immunoalkaline phosphatase procedures were employed using Fast Red as the chromogen to generate a red reaction product. The primary mouse monoclonal antibodies were as follows: RAM11 (specific for macrophages, diluted 1:50), HHF35 (specific for smooth muscle actin, diluted 1:50), and PC10 [specific for proliferating cell nuclear antigen (PCNA), diluted 1:200; all three purchased from DakoCytomation, Glostrup, Denmark] and N45.1 [specific for 8-hydroxy-2′-deoxyguanosine (8-OHdG), diluted 1:10; purchased from JaICA, Shizuoka, Japan]. The aortas were prepared as described in the preceding section. After hematoxylin–eosin staining, we chose six lesioned segments of each aorta. Image-Pro Plus 5.1 was used to measure the total aortic area and lesion areas (1 pixel/unit). For analysis using the antibodies against RAM11, HHF35, and 8-OHdG, the number of pixels in the positive area was divided by that in the lesioned area, and the result is expressed as a percentage. For PCNA, we determined the number of stained cells in the lesioned area, and the number in each 10^4 pixels was calculated for statistical analysis.

Data analysis and statistical analysis Data were analyzed using the Statistical Package for the Social Sciences 10.0 (IBM Corp., NY, USA). One-way ANOVA followed by least significant difference post hoc tests were used to determine the statistical significance of differences between the means.

RESULTS

Effects of Ctp on serum lipid levels Rabbits were orally administered 400 mg Ctp in distilled water or only water for three months, and the levels of serum lipids (TC, HDL-C, and TG) were measured and compared with those before Ctp administration. In comparison with the control group, which did not show any significant differences, TC levels in the Ctp group markedly decreased. There were no significant differences between the levels of HDL-C or TG between the control and Ctp groups during treatment (Fig. 1).

Macroscopic analyses of the areas in the aorta harboring atherosclerotic plaques Atherosclerotic plaques were present on the inner surfaces of the aortas in the control and Ctp groups (Fig. 2). The areas of atherosclerotic plaques in figure were indicated in red color by the image analysis software although the actual color was white. The plaques were white with irregular areas protruding into the aortic lumen. The distribution of atherosclerotic lesions was not uniform in the three areas, and severe involvement of the arch portion was observed, in contrast to only slight involvement of the thoracic and abdominal areas around branch sites and bifurcations. Neither superimposed thrombus nor ruptured plaque was observed on the vessel surface of either group.

Using the first and last intercostal artery as the boundaries, we subdivided the aorta into the arch, thoracic, and abdominal regions (Fig. 3A). The percentages of atherosclerotic plaques and the differences between the control and Ctp groups in each are shown in

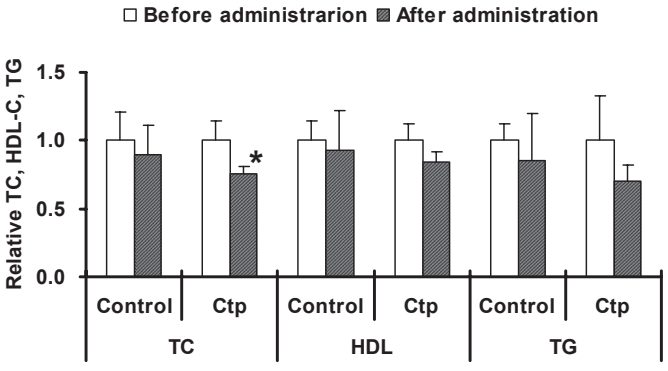

FIG. 1. Effects of Ctp on serum levels of TC, HDL-C, and TG. Each value represents the relative mean ± SD values of TC/HDL-C/TG levels of five animals. * $P < 0.05$ compared with the Ctp group before Ctp administration.

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