博士論文（要約）

Effects of Collagen Peptides on Gene Expression Related to Hair Cycle Activation in the Skin

（皮膚における毛周期活性化に関連する遺伝子発現に対するコラーゲンペプチドの影響）

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Introduction

The skin

In all mammals the skin is the largest organ that covers and protects the entire body. It forms not only a physical barrier against environmental changes but also a crucial immunobiological barrier against pathogen and ultraviolet light (1). Responsible for these functions are the two major compartments of the skin: the epidermis and the dermis (1) (Fig. 1). The epidermis is the outer part, forming the first and foremost barrier function of the skin (1). Populating the epidermis are four main layers of differentiated keratinocyte cells: the basal layer where cells with the most proliferating capacity reside, the spinous and granular layers where there are more differentiated cells, and the stratum corneum which faces the outside environment and which has terminally differentiated and enucleated cells (2). The predominant cell type in the dermis is fibroblast cells which produce and secrete components of the surrounding extracellular matrix (3). It is this extracellular matrix that makes the platform for cellular signals to approach the epidermis (3, 4). The integrity and functionality of the skin rely on the formation and remodeling of the extracellular matrix, the proliferation and differentiation of the keratinocyte cells, and the interactions between these two compartments (5). Thus, deregulation of these factors, for example by ultraviolet light or the intrinsic aging process, might cause defects to the skin (5, 6). In addition to these main compartments, the skin has appendages such as hair follicles, sebaceous glands and sweat glands (2). The skin also harbors local immune cells that can readily migrate to the surface in response to injury and infection (3).

Collagen

Collagen is a family of proteins that make up the largest part of the extracellular matrix throughout animal body (4). Of the twenty-eight known types of collagen proteins, collagen type I is the most abundant in the extracellular matrix of the skin (7). Not only responsible for providing the skin its moisture, elasticity and resilience, collagen is also a crucial attachment site for various signaling molecules either in the skin itself or migrated from inside the body to the skin when needed, for example in wound healing (8). However, the amount of collagen in the skin decreases with age, making the skin less flexible and more subject to environmental harmful factors (9).

Fortunately, several studies showed that oral intake of dietary collagen hydrolysates which are hydrolyzed products of collagen from various dietary sources can improve the skin conditions (discussed below). The use of collagen peptides as a dietary supplement for skin functions, nevertheless, has not gained complete approval, since it has been argued that collagen would eventually be degraded into free amino acids during digestion and would not reach the skin in a specific functional form. This argument has been proved untrue as specific collagen peptides resistant to intestinal peptidases have been found in the blood and several organs after oral intake of collagen hydrolysates (10-12).

Effects of dietary collagen peptides on the skin

It has been confirmed that oral intake of collagen peptides is safe in human and rat (11, 13). Early studies showed that collagen and collagen-derived peptides have antioxidant activities, which were found to be attributable to the high levels of proline, hydroxyproline and glycine residues in collagen (14). A few clinical studies showed that dietary collagen hydrolysates sufficiently reduced wrinkles, increased elasticity and moisture in aged skin (15, 16). Prolonged exposure to ultraviolet light triggers changes in the skin that are similar to the intrinsic aging process, hence the term photoaging (17). Experiments on mice have been focusing on the protective effects of oral administration of collagen hydrolysates against UV-induced damage in the skin. Several types of collagen hydrolysates from various sources, for example fish skin, chicken skin or porcine skin, have been used in such studies, all of which have shown that oral intake of collagen hydrolysates effectively suppressed UV-induced up-regulation of matrix metalloproteinases and UV-induced down-regulation of collagen production in mouse skin (18, 19). Not only do collagen hydrolysates impact the extracellular matrix in the dermis of the skin, but it was also found in a recent study that oral administration of collagen hydrolysates helps improve UV-induced loss of the skin barrier function (20). These findings suggest that although the antioxidant property cannot be completely excluded, it is not likely the sole mechanism in the action of dietary collagen hydrolysates in the skin.

Hair and hair follicles in the skin

Hair follicles are appendages of the skin that produce hair shafts which play an important role in temperature retention for all mammals (21) (Fig.2). They also have other less mentioned

functions. Hair is the means for sensing the environment, for social communication and for cleansing the surface of the skin (21). Hair follicles are responsible for forming and nourishing the hair, and for providing pigments to the hair and the skin (21). The epithelium of a hair follicle is composed of keratinocyte cells derived from the basal layer of the epidermis (21). Despite having the same origin, these cells undergo extensively diverse differentiation stages to form numerous specific layers of the hair follicle, each of which has a different function and shows a defined gene expression pattern (22). The mesenchyme of a hair follicle comprises of the dermal papilla which is a condensate of specialized fibroblasts in the dermis (22). The bulge region of a hair follicle contains a stem cell pool that can give rise to either hair follicle cells or epidermal cells upon activation by various stimuli and thus is important for skin and hair follicle regeneration (21-23).

The hair cycle

Along with the constant renewal of the epidermis, the hair follicles undergo a strictly regulated cycle which consists of three main stages: anagen - growth stage, catagen - regression stage, and telogen - rest stage (21-23). Many signaling pathways have been reported to play a role in the regulation of these stages in the hair cycle, but pathway crosstalk makes it complicated to understand the entire mechanism that governs the hair cycle. The relationship between the hair follicles and interfollicular epidermis further complicates the regulation. To date, however, the Wnt/ β-catenin signaling pathway is known to be an indispensible inducer and regulator of the anagen stage, whereas the bone morphogenetic protein pathway is the regulator of the telogen stage (21-24).

The molting cycle

The molting cycle is defined as the process in which aged or designated layers of the skin, feather, fur or hair shed out of the body to be replaced with newly formed ones. This process in hair follicle is also called exogen, which had been considered one of the stages of the hair cycle until it was observed that it can happen long after anagen has begun (25, 26). In hair biology nowadays exogen is referred to as an independent cycle in which a telogen hair shaft, irrespective of whether or not the next anagen has started, falls out of the body (25, 26). There is very little information on the regulation of exogen, although it is suggested that it might be

impacted by the regulation of anagen and vice versa (25, 26).

Studies on hair cycle-promoting reagents

Although loss of scalp hair is not likely to cause severe health problems, it has been a psychological concern in both men and women (27). The most common type of hair loss disease is the androgenetic alopecia which can occur in both men and women (27). There are currently two FDA-approved drugs for hair loss treatments, finasteride and minoxidil. Their effectiveness, however, has not been entirely approved, and it was found that they might cause side effects (28, 29). Over the past few years, attempts have been made in the search for natural compounds that have hair-cycle promoting effects and that are safe to use. In fact, numerous phytochemicals from plants and herbs have long been used as traditional remedies for hair loss in many countries (28-36).

Purpose of this study

Interest in the beneficial effects of dietary collagen hydrolysates on the skin has been growing remarkably over the last decade, probably because environmental insults have been increasing. There are more and more food products containing collagen hydrolysates that are claimed to benefit skin care. Although improvement in physical parameters of the skin has been shown in clinical studies using certain dietary products containing collagen hydrolysates, these claims are still not convincing because of the lack of molecular evidence. On which part of the skin collagen hydrolysates after oral intake might impact and how those impacts take place remain unclear. We performed both *in vivo* and *in vitro* experiments, identifying crucial gene expression changes induced by collagen peptides in the skin. Our data should prove essential in the search for the mechanisms of action of collagen hydrolysates.

Chapter 1 Inducing effect of oral administration of collagen peptides on expression of genes related to the hair cycle in mouse skin

Introduction

Despite the rising numbers of findings that oral intake of collagen peptides benefits the skin by increasing moisture, reducing wrinkles and protecting the skin from damage caused by ultraviolet light, the mechanism of action of collagen peptides remains elusive. Examination of changes in gene expression in the skin after oral administration of collagen peptides is an essential first step to explore the mechanism in question. In the experiment in my Master course, hairless mice under normal conditions (without UV irradiation) were divided into a control group and a collagen group. The latter was given collagen peptides orally every day for six weeks. Skin samples were eventually obtained from the mice and used for DNA microarray analysis.

In addition to identifying individual genes whose expression levels are altered by collagen intake, it is necessary to have an overview of the biological processes and pathways associated with these genes. Several databases and methods have been developed for this purpose. We used DAVID (Database for Annotation, Visualization and Integrated Discovery) for annotation analysis because it spans a wide range of well-developed annotation and pathway profiles such as GO and KEGG Pathways. Also, DAVID allows a selection of various statistical parameters which help us consider the gene groups from more than one aspect.

Materials and methods

Reagents

Porcine collagen peptides (SCP5200, mean molecular weight: 5000) was kindly given by Nitta Gelatin (Osaka, Japan). 5X PrimeScript^{TM} RT Master Mix and SYBR Premix Ex Taq (Tli RNase H Plus) were purchased from Takara (Shiga, Japan).

Animals

Six-week old female Hos:HR1 hairless mice were purchased from Japan SLC (Shizuoka, Japan). They were kept under conventional conditions for experimental animals (25°C, 12-hour light/ dark cycle). The mice had free access to normal diet (Labo MR Stock, pellet form, from Nosan Corp., Kanagawa, Japan) and sterile tap water during the acclimatizing period.

The mice were acclimatized to their housing conditions for six days before the experiment. They were then divided into two groups with similar average body weight (n = 5): a control group and a collagen peptides group (CP). The mice of the CP group were given *per os* 0.2 g of the porcine collagen peptides per kg body weight every day for six weeks, whereas those of the control group were given the same volume of sterile water. The mice were sacrificed after the administration period, and dorsal skin samples were taken for DNA microarray analysis. The animal experiment was performed in accordance with the guidelines of the University of Tokyo.

DNA microarray analysis

Same as described in my Master thesis. The Mouse Gene 1.0 ST Array (Affymetrix, California , USA) was used. Data were normalized by means of the robust multi-array average method.

Annotation analysis

DAVID (Database for Annotation, Visualization and Integrated Discovery) was used for annotation analysis following previously published instructions (37, 38). Briefly, the list of differentially expressed genes obtained from DNA microarray analysis was uploaded to DAVID, and enriched groups were selected with medium stringency. A group was considered interesting if fold enrichment was 1.5 or higher. Among the interesting groups, those that had enrichment score at least 1.3 were considered significant.

cDNA synthesis and real-time RT-PCR

The 5X PrimeScript™ RT Master Mix was used for cDNA synthesis following the manufacturer's protocol. Briefly, 0.5 μg total RNA (from individual mice of a group) was diluted in RNase-free water and 2 μL of the Master Mix was added to make a final 10 μL aliquot. The aliquots were incubated at 37°C for 15 min and 85°C for 5 sec. The heat block was programmed to keep the cDNA at 4°C after the incubation. The cDNA was then placed on ice for immediate use or kept at -30°C for later use.

Real-time RT-PCR was performed using a Roche LC96 LightCycler (Basel, Switzerland) following the manual. Briefly, 5 μL of the SYBR Premix Ex Taq was mixed with 0.2 μL each of forward primer and reverse primer and 3.6 μL of RNase-free water. The mixture was

loaded into a well of a PCR 96-well plate, and 1 μL of template cDNA was added to the mixture, followed by centrifugation at 1500 x g for 2 min. The plate was then loaded into the LightCycler and preheated to 95°C for 10 sec, followed by 40 cycles of three-step amplification: denaturation at 95°C for 5 sec, annealing at 56°C for 20 sec, and extension at 72°C for 20 sec. Hypoxanthine phosphoribosyltransferase (Hprt) was used as a reference gene for relative quantification. Relative fold changes were calculated using the ΔΔCt formula. Primer sequences are shown in Table 1 (39-41).

Statistical analysis

Statistical analysis for real-time RT-PCR results was done by means of Student's *t test*.

Results

Oral administration of collagen peptides induced up-regulation of two large sets of genes related to distinct biological functions

DNA microarray analysis revealed up-regulation of a number of keratin (Krt) and keratin-associated protein (Krtap) genes in the skin by oral administration of collagen peptides. These genes code structural proteins of hair follicles which are appendages of the skin. The keratin family is divided into epidermal keratins and hair keratins in terms of their expression site, and it is divided into acidic keratins and basic to neutral keratins in terms of their chemical feature (42). All of the keratin genes up-regulated in this experiment are hair-specific keratins, although the number of acidic keratins was higher than basic to neutral keratins (Table 2). The keratin-associated protein family is divided, according to their most abundant amino acid residues, into high glysine/ tyrosine KRTAP (rich in glycine and tyrosine), high sulfur KRTAP (rich in cysteine, threonine and proline), and ultra-high sulfur KRTAP (rich in cysteine) (43). The numbers of high glycine/ tyrosine, high sulfur and ultra-high sulfur Krtap genes up-regulated in this experiment were almost equal, suggesting that the effect of oral intake of collagen peptides did not depend on the content of amino acids provided from ingested collagen as materials required for synthesizing the protein products of these genes. Annotation analysis using DAVID further showed that these Krt and Krtap genes were associated with terms such as "intermediate filament cytoskeleton", "cytoskeletal part", "intracellular non-membrane-bounded organelle" (Table 3), emphasizing the relation of these up-regulated genes with cellular structure.

On the other hand, oral administration of collagen peptides also up-regulated expression levels of a number of olfactory receptor genes. These were found to be related to terms such as “sensory perception” and “G-protein coupled receptor protein signaling pathway” (Table 3). Since neither of these two functions has been described as benefits of collagen intake, this result might show new aspects in the effects of collagen on the skin.

The most enriched terms for differentially expressed genes induced by collagen peptides are related to skin and hair follicle functions

Analysis with DAVID identified “ectoderm development”, “epidermis development”, “molting cycle” and “hair cycle” as a significant group with an enrichment score of 1.4 (Table 4). Genes associated with these terms, G-protein-coupled receptor, family c, 5d (Gprc5d), small proline-rich protein 2a1 (Sprr2a1), keratin 27 (Krt27) and keratin-associated protein 16-7 (Krtap16-7), were up-regulated in the collagen peptide group, suggesting an induction of these processes. Real-time RT-PCR analysis for samples from individual mice showed that the expression levels of Gprc5d, Sprr2a1, Krt27 and Krtap16-7 as well as keratin-associated protein 15 (Krtap15), -8-2 (Krtap8-2) and -14 (Krtap14) were increased in the collagen peptides group, although this was not statistically significant (Fig. 3).

Discussion

Effects of dietary collagen peptides in the skin might involve changes in the cytoskeleton of skin cells, especially epidermis

The protective effects of collagen peptide intake against UV-induced skin damage have been extensively explored, and it has been suggested that these effects depend on collagen peptide-induced changes in the extracellular matrix in the dermis of the skin (18, 19). However, whether collagen peptide intake can also impact the extracellular matrix of normal skin is not well understood. In this experiment, hairless mice under normal conditions were given collagen peptides orally for six weeks and gene expression changes in the skin were determined. Interestingly, oral administration of collagen peptides significantly induced the expression levels of many keratin and keratin-associated protein genes (Table 2). Keratin proteins make up the filament network in keratinocyte cells of the epidermis and hair follicles (44). This filament network is stabilized by a large number of keratin-associated proteins (43). All of the keratin and

keratin-associated protein genes up-regulated by oral intake of collagen peptides are hair-specific, suggesting that collagen peptides might impact hair follicle and hair structure. There have been no reports on the impacts of collagen peptides on keratinocyte cells, probably because it is assumed that collagen peptides could only reach the dermis where they exert their effects on the extracellular matrix. However, we found that mRNA of the peptide transporter Pept1, which is suggested to be responsible for transportation of most oligopeptides, is expressed in keratinocyte cells at least at mRNA level (Le Vu Lan Phuong, master thesis, 2013). Recently the expression of this gene in the skin was confirmed (45). Therefore, it is reasonable that collagen peptides, after digestion in the intestinal tract, can be transported to the keratinocytes in the hair follicles in the skin and can alter the expression of the mentioned keratin and keratin-associated genes. Yet further analyses are needed to clarify whether these changes in gene expression actually impact the cytoskeletal structure of keratinocytes.

The involvement of the hair cycle and the molting cycle in the effects of collagen peptides

In addition to the intermediate filament-related terms, “hair cycle”, “molting cycle”, “ectoderm development”, and “epidermis development” were the significant enriched annotation terms associated with the effects (MT:functions?) of collagen peptides on the skin in this experiment (Table 4). While ectoderm development and epidermis development refer to processes of the epidermis from the formation before birth to the maturation after birth, hair cycle and molting cycle are processes that happen throughout the lifetime of an individual. Given that adult mice were used in this experiment, hair cycle and molting cycle are more relevant than the other two processes. The hairless mice used were Hos:HR1 which, according to the supplier and our own observation, still had hair follicles in their skin and they could grow very short and soft fur from time to time. The hair keratin proteins and keratin-associated proteins were found to be produced in the anagen stage of the hair cycle (43, 46), suggesting that the up-regulation of their expression by collagen peptides might link to an induction of anagen. On the other hand, molting cycle in this case might be related to either the shedding of the skin or of telogen-stage hair shafts. Both anagen of the hair cycle and exogen (shedding of telogen hair) were found to require keratinization, although the keratinized sites are different (47). Amongst the genes associated with the abovementioned terms, Gprc5d was found to be involved in keratinization of the hair shaft (48). Sprr2a1, on the other hand, codes one of the structural proteins of the

cornified envelope of the skin. Krt27 and Krtap16-7, like other Krt and Krtap genes up-regulated by collagen peptides, are structural components of hair follicle and hair. All in all, our data suggest that the effects of collagen peptides involve the hair cycle and/or molting cycle.

However, there is too little information on the regulation of the molting cycle to confirm whether oral administration of collagen peptides affects the hair cycle and the molting cycle by the same mechanism. Also, we could not observe hair growth or hair shedding in the hairless mice. Thus, it is necessary to use a more relevant experimental model to further understand the effects of collagen peptides.

Chapter 2 Inducing effect of prolyl-hydroxyproline (Pro-Hyp), a collagen dipeptide, on expression of genes related to the hair cycle in a co-culture of mouse skin cells

This chapter is under preparation for publication in an academic journal.

Chapter 3 Effects of collagen peptides on the hair cycle in vivo

This chapter is under preparation for publication in an academic journal.

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