論文の内容の要旨

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論文題目　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)

Introduction

The beneficial effect of dietary collagen peptides on the skin is a tantalizing topic that has been attracting more interest. It originated from the fact that collagen is the most abundant protein in the extracellular matrix in the dermis which is responsible for the elasticity, resilience and homeostasis of the skin. The amount of interstitial collagen decreases with onset of the aging process, making the skin more subject to damage. Although the loss of our own collagen cannot be compensated directly by dietary collagens from other species of origin, clinical studies have shown that oral intake of collagen hydrolysates from various sources can improve physical conditions of the skin effectively. Information on the effects of collagen hydrolysates on biological functions of the skin, nevertheless, is still limited. It had been argued that collagen cannot be distinguished from other dietary proteins: they are all degraded into free amino acids in the intestinal tract, thus not reaching the skin in any uniquely functional form. This was proved untrue, since oral intake of collagen hydrolysates was found to increase the concentration of collagen-specific hydroxyproline-containing di- and tripeptides in human blood. These peptides were also found to survive degradation by intestinal enzymes and serum enzymes *in vitro*. In addition, recent findings showed that these peptides can reach and remain in the skin for various periods of time. There are still debates, however, on how these peptides act once they are in the skin. In order to elucidate possible answers to this question, we approached gene expression analysis in both animal and cell culture models.

Chapter 1 Inducing effect of oral administration of collagen peptides on expression of genes related to hair cycle, molting cycle, epidermis development and ectoderm development in mouse skin

First, we performed an *in vivo* experiment using hairless mice, which is the most common model being used in this field because it provides easy observation of the skin. The mice were given porcine collagen peptides *per os* (0.2 g/ kg body weight daily) for a period of six weeks. Gene expression changes in their skin were determined by DNA microarray analysis. We found that a large number of hair keratin-associated protein genes and a group of hair-specific keratin genes were up-regulated in the collagen peptides group. All of these genes are known to code the hair follicle's and hair shaft's structural proteins that are produced in the growth phase of the hair cycle. Furthermore, annotation analysis by means of the Database for Annotation, Visualization and Integrated Discoveries (DAVID) revealed that the group of four annotation terms "hair cycle", "molting cycle", "epidermis development", and "ectoderm development" had an enrichment score of 1.4, suggesting that this group can be considered significant. Since adult mice were used in this experiment, epidermis development and ectoderm development are not likely to be applicable. On the other hand, hair cycle and molting cycle are continuous processes that happen throughout an animal's life. The genes associated with these terms in our differentially expressed gene list are G protein-coupled receptor family c, 5d (Gprc5d), keratin 27 (Krt27) and keratin-associated protein 16-7 (Krtap16-7). The expression levels of these genes were up-regulated in the collagen peptides group compared to those in the control group. Taken together, we considered that hair cycle and/or molting cycle were the biological processes influenced by the oral administration of collagen peptides in hairless mice.

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

In the previous chapter, we identified gene expression changes in whole skin induced by oral intake of collagen peptides *in vivo*. It is also essential to determine effects of collagen peptides on gene expression in individual compartments of the skin. For this purpose, we utilized a previously described co-culture system of mouse skin cells, since this system has an advantage over monolayer cell cultures: keratinocytes in this co-culture are able to form a differentiated and stratified skin-like layer, and fibroblasts are maintained in a three-dimensional matrix that

resembles their natural environment in the skin. Effects of the collagen-derived dipeptide prolyl-hydroxyproline (Pro-Hyp) on gene expression in this co-culture system were studied. Pro-Hyp has been suggested to be the most important component derived from collagen hydrolysates, owing to its highest abundance in the serum after ingestion of collagen hydrolysates and the stimulating effect it has on fibroblast cell growth. Interestingly, the expression levels of Krtap16-7, Krtap15, Krtap8-2 and Krtap14 in the skin-like layer were induced in the presence of Pro-Hyp. This effect was not seen when proline or hydroxyproline was used in place of Pro-Hyp, suggesting that the peptide was not degraded before exerting its function.

Since Krtap genes are related to the growing phase (anagen) of the hair cycle, we sought to determine effects of Pro-Hyp on regulators of anagen. The Wnt/ β-catenin signaling pathway is the most predominant pathway in the regulation of anagen, which prompted us to study the effects of Pro-Hyp on this pathway. By a PCR array analysis designed specifically for the Wnt/ β-catenin signaling pathway, we found that the expression of two of the Wnt ligand receptors, Fzd1 and Fzd6, were up-regulated by Pro-Hyp in the dermis equivalent. Meanwhile, that of Bmp4, a negative regulator of the Wnt/ β-catenin signaling pathway, was down-regulated by Pro-Hyp. In addition, the expression levels of β-catenin and Lef1, the transcription factors of this pathway, were up-regulated by Pro-Hyp. We adapted this co-culture system and, instead of fibroblasts only, we used a dermal cell preparation that included fibroblasts, developing hair follicle cells and other cell types. In this modified co-culture system, we found that protein level of β-catenin in the skin-like layer was increased by Pro-Hyp. Taken together, these results suggest that Pro-Hyp might positively influence the components of the Wnt/ β-catenin signaling pathway.

Chapter 3 Effects of oral administration of collagen peptides on the hair growth *in vivo*

Our previous *in vivo* experiment on hairless mice and *in vitro* experiment both hinted the inducing effect of collagen peptides on the expression of Krtap genes and the possible involvement of the hair cycle and/or molting cycle. The hair cycle is an activity of hair follicles with which to renew themselves through three basic phases: anagen (growth phase - when hair shafts are produced), catagen (regression phase), and telogen (resting, or quiescence phase). On the other hand, the molting cycle is a regular shedding off of the outermost layer of the skin or the telogen-phase hair shafts. There are no published experimental models for studying the molting cycle in mammals,

since the functions of it have not been well appreciated. Thus, we sought to explore the effects of oral intake of collagen peptides on the hair cycle using a conventional hair growth model. Nine-week old male Balb/c mice were shaved on the back, and they were given porcine collagen peptides *ad libitum* (2% in a powder diet) for fifteen days. Under these conditions, however, we did not find a promoting effect of collagen intake on actual hair growth. Average hair thickness was lower in the collagen peptides group compared to that in the control group. The expression level of Gprc5d was slightly decreased in the collagen peptide group, whereas those of Sprr2a1, Krt27 and Krtap16-7 were increased, although these changes were not statistically significant.

Conclusion

Our study provided the first line of evidence that oral administration of collagen peptides has regulatory effects on gene expression in the skin. We also showed that Pro-Hyp influences gene expression of mouse skin cells in the co-culture system, supporting the hypothesis that Pro-Hyp is the key peptide in the effects of collagen-derived peptides on the skin, at least for the Krtap genes that were found up-regulated in both the *in vivo* and *in vitro* experiments. The possible involvement of hair cycle and/or molting cycle gave a new insight to the effects of collagen peptides on the skin. In our second *in vivo* experiment, we could not find hair growth-promoting effects of oral administration of collagen peptides. We cannot exclude, nevertheless, the possibility that the conditions used in this experiment were not optimal. Also, the effects of collagen peptides intake on the molting cycle should be taken into account. It will be interesting to clarify whether collagen peptides act on these processes separately or jointly.

Publication

1. Le V. P., Takatori R, Iwamoto T., Akagi Y., Satsu H., Totsuka M., Chida K., Sato K., Shimizu M.. Effects of Food-Derived Collagen Peptides on the Expression of Keratin and Keratin-Associated Protein Genes in the Mouse Skin, *Skin Pharmacology and Physiology*, Vol. 28, pp. 227-235, 2015.