Contents, structure, and functions of collagen-derived peptides in human blood after ingestion of collagen hydrolysate and gelatin

Tomoko Asai

2020

Contents

General Introduction

Collagen is a major proteinaceous component of the extracellular matrix and making up approximately 25–35% of the total protein of animals. Collagen forms molecular family consisting of different gene products. At least 28 types of collagen have been identified in mammals, which are designated by Roman numerous such as type I –type XXVIII.[1] Type I collagen is the most abundantly distributed in tissues except for cartridge. Collagen molecule consists of three subunits, referred to α chains. The three subunits with characteristic repeating motives of glycine (Gly)-X-Y, where X and Y are often occupied by proline (Pro) and hydroxyproline (Hyp), form triple helical structure. The triple helical structure resists most of the protease except for collagenases such as matrix metalloproteinases (MMP)-1 and 8.[2] The triple helical structure of collagen collapses into random coiled structure by heat-treatment with water.[3] The denatured form of collagen is referred to as gelatin.[3] Gelatin is degraded into low molecular peptides (~0.3–8 kDa) by protease, which is referred to collagen hydrolysate, gelatin hydrolysate, or collagen peptide.[4] The collagen hydrolysate is highly soluble compared to gelatin and loses gel-forming ability.[5] The collagen hydrolysate is prepared on an industrial scale and used as a functional food ingredient.[5] As start material of collagen hydrolysate, fish skin and scale and animal skin, bone, and tendon are used.[5] Recently, market of collagen hydrolysate is expanding around the world, the annual sales of final products in the world is approximately 5 billion US dollar.[6]

Some human trials using placebo controls have demonstrated that ingestion of collagen hydrolysate (2.5–10 g per day) show beneficial effects as follows. Ingestion of 10 g collagen hydrolysate per day for 4–6 months enhances healing of pressure ulcers[7,8] and attenuates the symptoms of osteoarthritis.[9,10] Ingestion of 2.5 g collagen hydrolysate for 2 months improves various skin conditions, such as reduction of wrinkle volume, increase of elasticity and skin moisture in women subjects with specific ages.[11,12]

However, it was assumed before 2005 that orally administered collagen peptides did not exert special effects, as collagen peptides were believed to be degraded into amino acids during digestion and absorption processes. This situation was dramatically changed by findings of food-derived collagen peptides in human blood at 2005.[13] Ingestion of collagen hydrolysate (2–20 g) increases peptide forms of Hyp, (i.e. collagen peptide) in human peripheral blood plasma to 20–200 μM.[13–17] To date, the presence of more than 10 food-derived collagen peptides in human blood has been reported as follows; prolyl-hydroxyproline (Pro-Hyp), hydroxyprolyl-glycine (Hyp-Gly), alanyl-hydroxyproline (Ala-Hyp), isoleucyl-hydroxyproline (Ile-Hyp), leucyl-hydroxyproline (Leu-Hyp), phenylalanyl-hydroxyproline (Phe-Hyp), glutamyl-hydroxyproline (Glu-Hyp), prolyl-hydroxyprolyl-glycine (Pro-Hyp-Gly), glycyl-prolyl-hydroxyproline (Gly-Pro-Hyp), alanyl-hydroxyprolyl-glycine (Ala-Hyp-Gly), and serinyl-hydroxyprolyl-glycine (Ser-Hyp-Gly). Pro-Hyp and Hyp-Gly are the main food-derived collagen peptides found in human blood after ingestion of collagen hydrolysate.[13–17] These peptides have been reported to resist to peptidase digestion in human plasma.[18] In vitro studies have shown that these food-derived collagen peptides enhance the growth of mouse skin fibroblast attached on collagen gel[18,19] and the production of glycosaminoglycan by fibroblasts [15] and chondrocytes,[20] which have been associated with the beneficial effects of ingestion of collagen hydrolysate. Pro-Hyp is also generated by degradation of endogenous collagen in tissues with inflammation[21] and wound healing site of skin,[22] whereas the generation of Pro-Hyp is suppressed in db/db mice,[22] type 2 diabetes model, which shows delayed wound healing. Therefore, Pro-Hyp might play important role in the wound healing process.

Recently, collagen hydrolysates with different molecular size are prepared from many start materials.[23–25] Contents of collagen peptides in human blood after ingestion of these collagen hydrolysates have been examined. [24–26] Animal and fish meats in daily meal are also containing collagen or gelatin. However, there is no data about the levels of collagen

peptide in blood after ingestion of these meats in daily meal. In chapter 1, amount of ingested collagen by consuming animal and fish meats in Japanese daily dishes were examined. In addition, contents and structure of food-derived collagen peptides after ingestion of cooked fish meat rich in gelatin were compared to those after ingestion of collagen hydrolysate.

As mentioned above, Pro-Hyp triggers growth of fibroblasts attached on collagen gel.[18,19] However, inconsistent results were obtained, when different preparation of fetal bovine serum (FBS) was used. To solve this problem, presence and contents of low molecular weight collagen peptides in FBS and its effects on growth of fibroblasts were examined in chapter 2.

It has been demonstrated that response of fibroblasts to Pro-Hyp differs between primary cultured cells and sub-cultured ones.[19] In addition, ingestion of collagen hydrolysate can enhance wound healing without induction of excess fibrosis in normal tissues.[8] These facts suggest that presence of the Pro-Hyp responding and non-responding fibroblasts. In chapter 3, the Pro-Hyp responding fibroblasts were identified on the basis of incorporation of fluorescein isothiocyanate (FITC)-labeled Pro-Hyp and expressing mesenchymal stem cell marker, p75 neurotrophin receptor (p75NTR).

This study demonstrates that ingestion of gelatin rich cooked meat increases food-derived collagen peptides in blood and that Pro-Hyp specifically enhances growth of fibroblasts with only specific condition, which could explain why ingestion of collagen hydrolysate enhances wound healing without inducing adverse effects on normal tissue.

Chapter 1

Amount of collagen in the meat contained in Japanese daily dishes and the collagen peptide content in human blood after ingestion of cooked fish meat

1.1 Introduction

Collagen is a major component of the extracellular matrix in animals and has a triple helix structure. Triple helical structure resist to most proteases except for collagenase. Heat treatment causes the triple helix structure of collagen to collapse into a random coiled structure, referred to as gelatin.[3] Gelatin is prepared from the bones, skin, and tendons of animals and the scales, skin, and bones of fish.[3] Soluble gelatin can be easily degraded by most endoproteinases. Enzymatic hydrolysate of gelatin is more soluble than gelatin and is referred to as collagen hydrolysate or collagen peptide.[5] Some human trials have demonstrated that ingestion of collagen hydrolysate has beneficial effects, which have been reported as both subjective and objective effects, such as enhanced healing of pressure ulcers,[7,8] attenuation of the symptoms of osteoarthritis,[9,10] and improvement of skin condition.[11,12] A lot of human trials have demonstrated that the levels of some collagen derived di- and tri- peptides, such as Pro-Hyp, Hyp-Gly, Pro-Hyp-Gly, and others are increased in human blood after ingestion of collagen hydrolysate. In vitro studies have shown that these food-derived collagen peptides enhance the growth of fibroblast,[18,19] and the production of glycosaminoglycan by fibroblasts[27] and chondrocytes,[28] which is associated with the beneficial effects of collagen hydrolysate ingestion.

Animal and fish meat contain collagen, and at least part of the collagen in such meat can be converted to gelatin during cooking. The gelatin in cooked meat can be degraded by digestive proteases. Therefore, the levels of some collagen peptides may be also increased in human blood after ingestion of cooked meats.

The collagen contents in the muscles of animals, birds, and fish free from large connective tissues has been determined.[29–31] However, the meat in food dishes frequently includes skin as well as large intra- and inter-muscular connective tissues. Therefore, it is difficult to estimate the amount of collagen ingested in a meal based on the available data. Furthermore, there are no data on the structure and content of the collagen peptides present after ingestion of meat. To address these issues, the collagen contents of the fish, chicken, pork, and beef meat contained in one serving of a Japanese daily dish were determined in the present study. In addition, the structure and content of the collagen peptides present in human blood after ingestion of a collagen-rich meat were evaluated and compared to those after ingestion of a collagen hydrolysate.

1.2 Materials and Methods

Meat Preparation

Fish meat was selected based on the frequency of inclusion in Japanese daily meals according to the Japan National Health and Nutrition Survey Report.[32] Blue shark (Prionace glauca) meat was also included in this study, since shark and ray meats are rich in collagen and are traditionally consumed in a specific area of Japan.[33] Generally, small-sized fish are gutted or headed and used in a round form. Larger fish are usually filleted and cut into single servings. Whole fillets and fillet cuts are usually grilled, steamed, or simmered with the skin on. Sliced raw meat (*sashimi*) is generally prepared from skinned fillets.[34] Eel is usually butterflied, and the whole fillet with the skin is steamed and then grilled with seasonings. Relatively large butterflied or cut fillet of eel is typically used for

a single serving.[34] Shark is generally skinned, as the skin has a hard layer. Then, the skinned shark meat is usually cut into fillets and chunks for one serving. Squid is cut into the mantle and arm meat portions. The arm meat is usually fried, grilled, or simmered, and the mantle meat with the skin is also fried, grilled, or simmered. Skinned mantle meat is also prepared as *sashimi*.[34]

Japanese horse mackerel (*Trachurus japonicas*), chub mackerel (*Scomber japonicas*), red sea bream (*Pagrus major*), chum salmon (*Oncorhynchus keta*), Japanese eel (*Anguilla japonica*), and Japanese common squid (*Todarodes pacificus*) were collected as whole fish from local markets and cut into single servings by an expert chef from Kyoto Culinary Art College (Kyoto, Japan) as shown in Figure 1-1. Blue shark (*Prionace glauca*) was collected and cut into a single serving by an expert chef in Chuka Takahashi (Miyagi, Japan) using the parts shown in Figure 1-1. Beef meat (rib, flank, chuck, tender loin, and tendon), pork meat (loin, belly, and ham), and chicken meat (thigh and breast) were obtained from a local wholesaler. The beef, pork, and chicken meats were cut into single servings for Japanese daily dishes by the aforementioned expert chef. Typically, approximately 100 g of meat is used for a single serving in Japan.

The whole cuts of meat were freeze-dried with an FDU-1200 (Eyela, Tokyo, Japan) and crushed in a blender (WB-1; Osaka Chemical, Osaka, Japan).

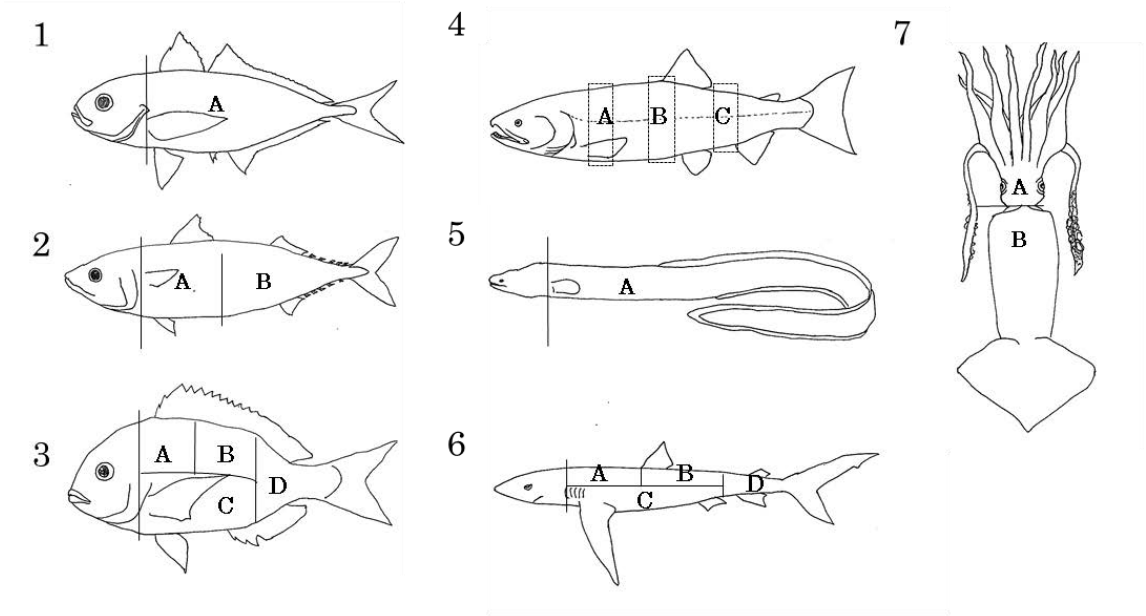

Figure 1-1. The fish parts used by the chefs to prepare the meats. 1, Japanese horse mackerel (A, whole trunk); 2, chub mackerel (A, anterior part of the trunk; B, posterior part of the trunk); 3, red sea bream (A, anterior portion of the dorsal part of the trunk; B, posterior portion of the dorsal part of the trunk; C, ventral part of the trunk; D, tail), 4, chum salmon (A, anterior part of the trunk; B, middle part of the trunk; C, posterior part of the trunk); 5, Japanese eel (A, whole trunk); 6, blue shark (A, anterior portion of the dorsal part of the trunk; B, posterior portion of the dorsal part of the trunk; C, ventral part of the trunk; D, tail); 7, Japanese common squid (A, arm; B, mantle).

Chemicals

A standard mixture of amino acids (Type H), acetonitrile (HPLC-grade), trimethylamine, phenyl isothiocyanate (PITC), piperidine, 4-methylmorpholine, N, N-dimethylformamide, t-butyl methyl ether, and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). 9-Fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives, Fmoc glycine-bond p-alkoxybenzyl alcohol (Alko) resin, proline and hydroxyproline-bond 2-chlorotrityl chloride (Barlos) resin, 1H-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), and 1-hydroxybenzotriazole (HOBt) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Pro-Hyp and Hyp-Gly were obtained from Bachem (Budendorf, Switzerland). L-Hydroxyproline (Hyp), porcine pepsin, and pancreatin were purchased from Nacalai Tesque (Kyoto, Japan). Leucine aminopeptidase and carboxypeptidase A were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade or better.

Human Study

The human study was performed according to the Declaration of Helsinki under the supervision of medical doctors and was approved by the experimental ethical committee (201510-23-CTS001) of CX Medical Japan (Tokyo, Japan). Collagen hydrolysate from fish scales (Ixos gel HDL-50SP; Average molecular weight, 4769 Da) was a kind gift from Nitta Gelatin (Osaka, Japan).

Skinned and deboned tail shark meat (part D) was simmered with seasonings. Hyp was not detected in the seasoning. The volunteers (average age 31, average body weight 68.1 kg) were informed of the study objective and the potential risks of ingesting the samples and blood collection. After fasting for 12 h, six healthy male volunteers ingested 200 g of cooked shark tail meat, which contained 13.5 ± 1.0 g of collagen. Approximately

10 mL of venous blood was collected from the cubital vein before ingestion and 30, 60, 120, 240, and 360 min after ingestion. Plasma was prepared and mixed with three volumes of ethanol. Then, the supernatant was collected by centrifugation at 1,000 × *g* for 10 min and stored at -80 °C until use. After a 2-week washout period, the same volunteers ingested 13.5 g of collagen hydrolysate dissolved in 100 mL of water. Then, blood was collected and processed as described above.

Hot Water Extraction of the Collagen in Shark Meat

Freeze-dried powdered raw and cooked shark tail meat (1 g) were suspended in 10 mL of water and heated at 100 °C for 10 min. Then, the suspension was centrifuged at 10,000 × *g* for 5 min. The residue was treated as described, twice, and the supernatant was collected and used as the hot water-soluble fraction.

In Vitro Protease Digestion

Freeze-dried powdered cooked shark tail meat and collagen hydrolysate (1 g) were suspended in 150 mL of 0.1 M HCl. Then, 10 mg of pepsin was added and incubated at 37 °C for 3 h. Next, the reactant was mixed with 10 mL of 1 M Tris-HCl buffer (pH 8.0), and the pH was adjusted to 8.0 by the addition of 1 M NaOH. Then, 40 mg of pancreatin was added and incubated for 24 h. The pepsin-pancreatin digest was brought up to 200 mL by the addition of water and clarified by centrifugation at 3,000 × *g* for 10 min. Leucine aminopeptidase (2.5 units) and carboxypeptidase A (3 units) were added to aliquots of the digests (500 μL) and incubated at 37 °C for 24 h. The same amount of freeze-dried powdered cooked shark tail meat was treated in the same manner without enzymes.

Size Exclusion Chromatography

Collagen hydrolysate was dissolved in water to yield a 5 mg/mL solution. The in vitro protease digests (500 μL) were freeze-dried and dissolved in 250 μL of 10% acetonitrile. These solutions were clarified by passage through a Cosmonice filter (0.45 μm, Nacalai Tesque). The filtrate (approximately 200 μL) was subjected to size exclusion chromatography (SEC) using a Superdex Peptide 10/300 GL size exclusion column (GE Healthcare, Buckinghamshire, UK). Peptides were eluted with 0.1% formic acid containing 10% acetonitrile at 0.5 mL/min, and fractions were collected every 1 min.

Aliquots of the SEC fractions were directly injected into an electron spray ionization mass spectrometer (LCMS-8040; Shimadzu, Kyoto, Japan). The molecular weights of the peptides in the SEC fractions were estimated based on the mass to charge ratio (*m/z*).

Amino Acid Analysis

Freeze-dried powder (10–1000 mg) was hydrolyzed in vacuo with 1 mL of 6 M HCl in a vial with a valve (Pierce vial, 40 mL; Thermo Fisher Scientific, Waltham, MA) at 150 °C for 1 h. The hydrolysate was brought up to 50 mL with water. The amino acids in the hydrolysate were analyzed according to the method of Bidlingmeyer et al. [35]

The Hyp contents were converted to collagen contents by multiplying by the coefficient (collagen weight/Hyp weight), which was obtained from the amino acid composition of collagen for each species.[3,36–41]

Determination of the Food-derived Collagen Peptides in Human Blood Plasma

The Hyp-containing peptide contents were evaluated by amino acid analysis as described previously.[13] To evaluate the content of each collagen peptide in plasma, liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode was performed using an LCMS-8040 (Shimadzu) and a high pressure binary gradient HPLC (LC20 system; Shimadzu). Eleven Hyp-containing

peptides (Pro-Hyp, Hyp-Gly, Ala-Hyp, Ile-Hyp, Leu-Hyp, Phe-Hyp, Glu-Hyp, Pro-Hyp-Gly, Gly-Pro-Hyp, Ala-Hyp-Gly, and Ser-Hyp-Gly), which have been observed in human plasma after ingestion of collagen peptides,[13,16,42] were purchased or synthesized by the Fmoc strategy using an automatic peptide synthesizer (PSSM-8; Shimadzu) according to the supplier's protocols. The peptides were derivatized with AccQ as described below and used to optimize the MRM conditions by using LabSolutions Version 5.65 (Shimadzu).

For the analysis, 100 μL of the 75% ethanol-soluble fraction of human plasma was dried in a 1.5 mL tube. Then, 20 μL of 20 mM HCl, 20 μL of a 0.3% AccQ acetonitrile solution, and 60 μL of 50 mM sodium borate buffer (pH 8.8) were added to the residue and reacted at 50 °C for 20 min. The reaction mixture was diluted with 100 μL of 5 mM sodium phosphate buffer (pH 7.4) containing 10% acetonitrile. Aliquots (10 or 20 μL) were injected into the LC-MS/MS, and AccQ peptides were resolved on an Inertsil ODS-3 column (2.5 μm, 2.1 mm × 250 mm; GL Science, Tokyo, Japan) equilibrated with 0.1% formic acid (solvent A). Binary gradient elution was applied with solvent A and solvent B (0.1% formic acid containing 80% acetonitrile) at a flow rate of 0.2 mL/min. The gradient profile was as follows: 0–15 min, 0–50% B; 15–20 min, 50–100% B; 20–25 min, 100% B; and 25–35 min, 0% B. Detection was performed by MRM in positive ion mode. The sample was spiked with standard peptides (5 pmol). The recoveries of the spiked standards were calculated. To correct for a matrix effect on the ionization of sample peptides, the value of each peptide was divided by the recovery of each standard.

Statistical Analyses

The differences between the means were evaluated by one-way analysis of variance (ANOVA) and followed by Tukey's multiple comparison test for post-hoc analysis using GraphPad Prism Version 6.04. (GraphPad Software, San Diego, CA). The differences between two groups were compared using Student's *t*-test.

1.3 Results and Discussion

Amount of Collagen in the Meat Contained in One Serving of a Japanese Dish

Skin and intramuscular connective tissues, such as myocommata and epimysium, which cover the muscles of fish and animals, respectively, are rich in collagen. The skin and intramuscular connective tissue remained as relatively large particles on the freeze-dried meats even after milling, resulting in the generation of non-homogenous powders. To minimize deviations in collagen content due to sampling, relatively large amounts of powder should be used. My preliminary experiments showed that the coefficient of variation in Hyp content in the same sample was less than 5% (n = 4) when 1 g of freeze-dried powder was used for the amino acid analysis. Thus, 1 g of freeze-dried sample was used to determine Hyp contents.

There are a few reports on the collagen contents in fish and animal meat.[29,43] However, in these studies, the collagen contents in meat that was free of skin and from specific parts of body were evaluated, and the amount of meat contained in one serving of a daily meal was not considered. To evaluate the actual collagen intake from a daily Japanese meal, the amounts of collagen in the meat included in one serving of a Japanese daily dish prepared by expert chefs were determined in the present study. The weights of the meats in one serving of Japanese dishes prepared by expert chefs are shown in Table 1-1. The weights of the fish meats were ~50–200 g depending on the fish species and cooking method, and the weights of the animal meats were ~80–130 g.

The contents and amounts of collagen in the meat contained in a single serving of a Japanese dish varied from 0.3 to 8.5 g/100 g wet tissue and 0.2 g to 13.3 g, respectively (Table 1). A one serving fillet with skin of Japanese horse mackerel, chub mackerel, red sea bream, or chum salmon contained 0.7–1.2 g of collagen. For skinned red sea bream, the collagen content decreased by half when compared to the fish meat with skin. One serving of Japanese eel contained high amounts of collagen (~13 g) due to the thick skin

and intramuscular connective tissue and the large size of the serving (approximately 200 g). For blue shark, the cuts of skinned fillets (A, B, and C) contained 0.7 g of collagen. Chucked tail meat (D) contained 13.3 g of collagen, which was the highest value observed among the examined preparations of skinned meat. One serving of animal, chicken, and squid meat contained ~1–2 g of collagen, except for tender loin (0.41 g) and tendon (7.56 g). Chicken meat with skin contained ~1.8 times higher amounts of collagen than skinned meat. These results suggest that 0.2–5 g of collagen can be ingested by consuming two servings of meat a day. This value is close to the average intake of collagen reported by Noguchi et al.[43] (1.9 g). However, more than 10 g of collagen can be ingested in a single serving of a collagen-rich meat such as stewed beef tendon, grilled eel, or simmered shark tail.

Table 1-1. Weights of the Meats Prepared for One Serving of a Japanese Daily Dish by the Expert Chef and the Amounts of Collagen in the Meats.

species	parts	weight of the meat (g)	collagen amount in the meat (g)	collagen content in the meat (g/100 g)
Japanese horse mackerel (*Trachurus japonicas*)	A*	98.8 ± 12.6	1.64 ± 0.25	1.67 ± 0.24
chub mackerel (*Scomber japonicas*)	A*	65.5 ± 2.4	0.75 ± 0.04	1.14 ± 0.07
	B*	66.9 ± 2.10	0.70 ± 0.07	1.07 ± 0.13
red sea bream (*Pagrus major*)	A	54.7 ± 1.7	0.29 ± 0.07	0.53 ± 0.12
	B	51.3 ± 2.0	0.21 ± 0.02	0.40 ± 0.05
	C	52.2 ± 1.7	0.25 ± 0.06	0.49 ± 0.14
	D	39.7 ± 2.9	0.34 ± 0.03	0.85 ± 0.05
	A*	58.3 ± 2.2	0.79 ± 0.07	1.36 ± 0.23
	B*	52.2 ± 2.1	0.67 ± 0.02	1.28 ± 0.29
	C*	57.9 ± 3.5	0.84 ± 0.06	1.45 ± 0.24
	D*	44.9 ± 2.8	0.86 ± 0.03	1.90 ± 0.59
chum salmon (*Oncorhynchus keta*)	A*	70.3 ± 11.9	1.16 ± 0.27	1.74 ± 0.45
	B*	75.3 ± 3.1	1.17 ± 0.29	1.63 ± 0.41
	C*	74.4 ± 2.8	1.41 ± 0.27	1.89 ± 0.33
Japanese eel (*Anguilla japonica*)	A*	193.8 ± 10.1	13.09 ± 2.56	6.74 ± 1.15
blue shark (*Prionace glauca*)	A	55.9 ± 1.3	0.71 ± 0.17	1.27 ± 0.29
	B	57.0 ± 1.0	0.65 ± 0.06	1.14 ± 0.09
	C	57.2 ± 1.0	0.63 ± 0.05	1.11 ± 0.09
	D	166.9 ± 25.9	13.3 ± 2.96	8.48 ± 0.71
cattle (*Bos taurus*)	Rib	120.3 ± 10.5	1.64 ± 0.37	1.35 ± 0.20
	Flank	105.6 ± 2.8	0.97 ± 0.12	0.91 ± 0.09
	Chuck	120.5 ± 3.9	1.32 ± 0.10	1.09 ± 0.11
	Tender loin	130.0 ± 8.6	0.41 ± 0.06	0.32 ± 0.04
pig (*Sus scrofa domesticus*)	Tendon	101.2 ± 2.3	7.56 ± 3.61	7.49 ± 3.62
	Loin	100.6 ± 2.3	1.90 ± 0.32	1.89 ± 0.36
	Belly	80.2 ± 4.9	1.64 ± 0.50	2.08 ± 0.73
	Ham	78.8 ± 1.2	1.23 ± 0.34	1.56 ± 0.34
chicken (*Gallus gallus domesticus*)	Thigh	112.3 ± 2.5	1.39 ± 0.04	1.24 ± 0.05
	Thigh*	123.4 ± 4.2	2.52 ± 0.12	2.05 ± 0.13
	Breast	118.3 ± 8.1	0.80 ± 0.34	0.67 ± 0.23
Japanese common squid (*Todarodes pacificus*)	A	101.4 ± 7.8	1.17 ± 0.17	1.16 ± 0.18
	B	50.2 ± 5.5	0.72 ± 0.12	1.43 ± 0.16

Values are mean ± S.D. of three determinations. Asterisks (*) indicate meat with skin.

Content and Structure of the Collagen Peptides in Human Blood Plasma

After ingestion of the collagen hydrolysate, the hydroxyproline (Hyp)-containing di- and tri-peptides in human blood were increased. However, there are no data on the structure and content of the Hyp-containing peptides in human blood after ingestion of collagen-containing meats. Cooked skinned chuck tail meat from blue shark (part D) was used in the human study, since it contained the highest amount of collagen. Collagen hydrolysate was used as a positive control. One serving (200.1 ± 7.0 g) of cooked shark meat contained 13.5 ± 0.7 g of collagen. The concentration of the free Hyp and Hyp-containing peptide in human plasma before and after ingestion of collagen hydrolysate (13.5 g) and cooked shark meat are shown in Figure 1-2. After ingestion of the cooked shark meat, both the free Hyp and Hyp-containing peptide reached maximum levels of ~45 and 14 μM in plasma, respectively, at 60 min. These values were approximately one-half and one-fifth of the levels observed after ingestion of 13.5 g of collagen hydrolysate (~90 and 70 μM, respectively). The area under the curve (AUC) of the free Hyp and Hyp-containing peptide after ingestion of cooked shark meat were 61% and 28% of those detected after ingestion of collagen hydrolysate (Table 1-2).

Nine collagen-derived peptides: Pro-Hyp, Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, Ser-Hyp-Gly, Ile-Hyp, Leu-Hyp, and Phe-Hyp were significantly increased in plasma after ingestion of cooked shark meat, while no significant increases in Glu-Hyp and Gly-Pro-Hyp were observed (Figure 1-3). The peptides with the first and second highest levels in plasma after ingestion of cooked shark meat and collagen hydrolysate were Pro-Hyp and Hyp-Gly. The maximum levels of most peptides after ingestion of cooked shark meat were significantly lower than those after ingestion of collagen hydrolysate except for Hyp-Gly, Pro-Hyp-Gly, and Ile-Hyp ($p < 0.05$; Figure 1-3). The AUC of the nine peptides after ingestion of collagen hydrolysate and cooked shark meat are summarized in Table 1-2. The AUC of the peptides after ingestion of cooked shark meat were 17–38% of those after ingestion of collagen hydrolysate, and most were

significantly lower ($p < 0.05$) except for Hyp-Gly, Pro-Hyp-Gly, and Ile-Hyp. The sum of the peptide contents in plasma as evaluated by LC-MS/MS accounted for approximately 70% of the total Hyp-containing peptides as evaluated by amino acid analysis. The ratio of Hyp-Gly to Pro-Hyp in plasma after ingestion of cooked shark meat was significantly higher than that after ingestion of collagen hydrolysate. It has been reported that Hyp-Gly and Pro-Hyp are resistant to the exopeptidases in blood.[18,19] Therefore, endoproteinase might cleave the peptide bonds between Hyp and Gly or Pro and the Hyp residues in larger peptides. The present data suggest that human digestive endoproteinase preferentially cleave the peptide bond between the Pro and Hyp residues compared to the bacterial endoproteinase used to generate the collagen hydrolysate. It has been demonstrated that Hyp-Gly enhances the growth of fibroblasts on collagen gel to a greater extent than Pro-Hyp.[18] Therefore, ingestion of cooked meat containing collagen/gelatin might have the beneficial effects similar to those observed after ingestion of collagen hydrolysate, despite the lower collagen peptide contents observed in plasma after ingestion.

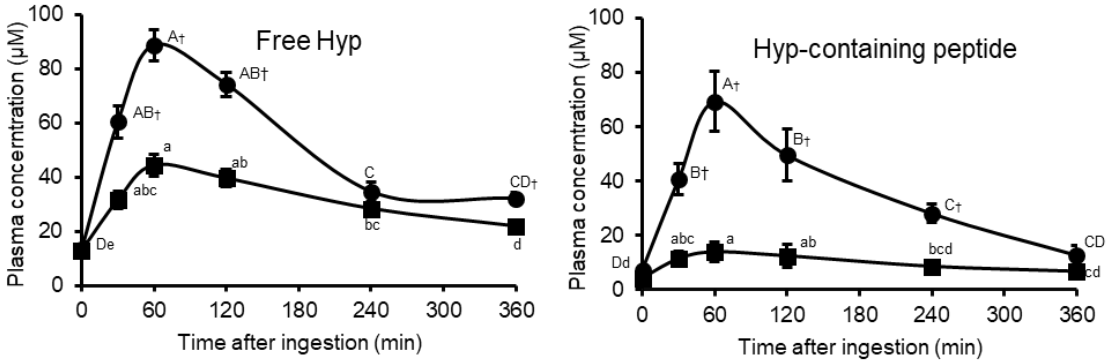

Figure 1-2. Free Hyp and Hyp-containing peptide contents in human blood plasma after ingestion of collagen hydrolysate (●) and cooked shark tail meat (part D; ■). Data are mean ± S.E. (n = 6). Different letters on data points indicate significant differences ($p < 0.05$) between the contents at different time points after ingestion. Daggers (†) indicate significant differences between the two groups (tail meat and collagen hydrolysate).

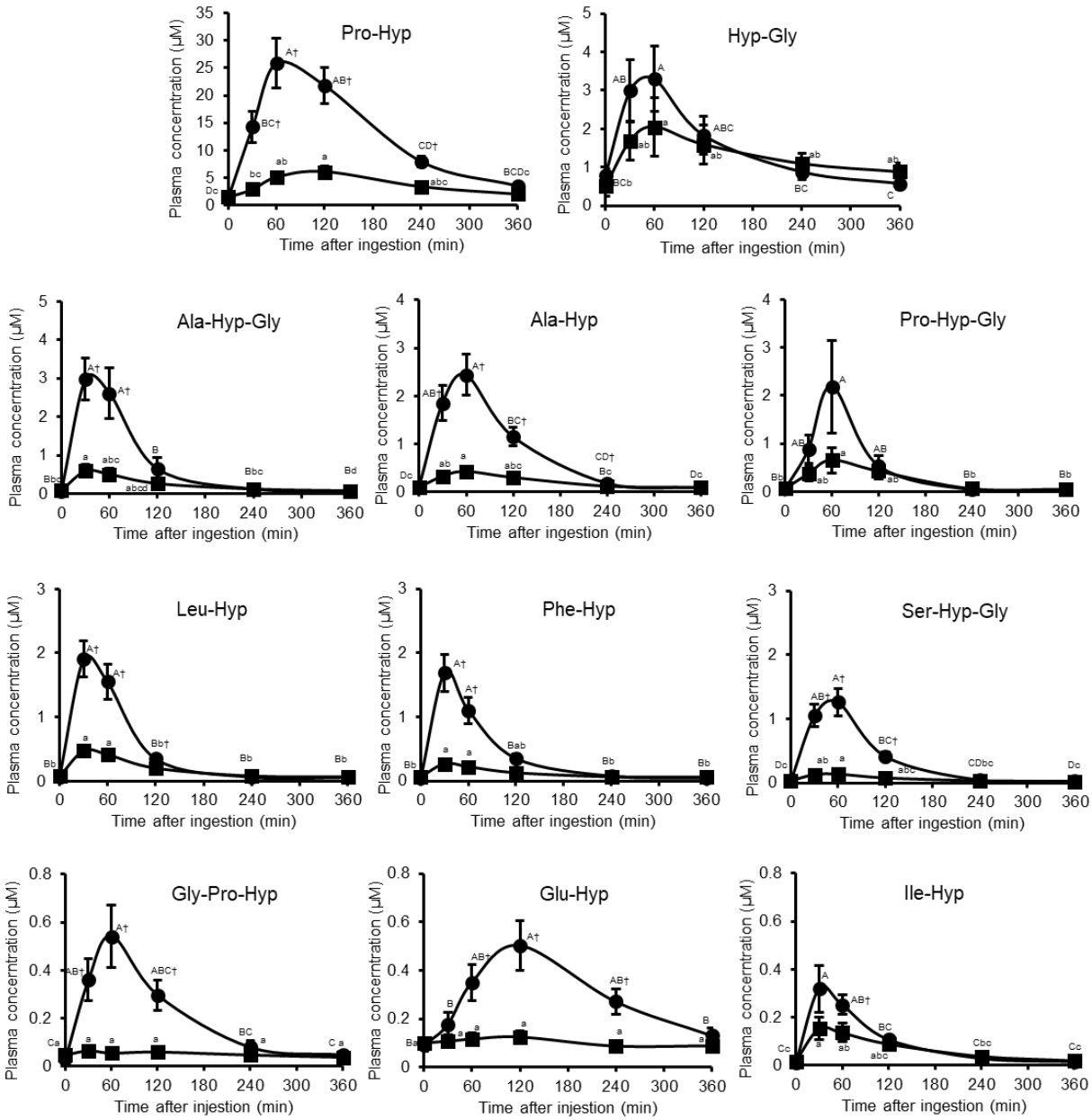

Figure 1-3. Collagen peptide contents in plasma after ingestion of collagen hydrolysate (●) and cooked shark tail meat (part D; ■). Data are mean ± S.E. (n = 6). Different letters on data points indicate significant differences ($p < 0.05$) between different time points after ingestion. Daggers (†) indicate significant differences between the two groups.

Table 1-2. Area Under the Curve (AUC) of the Collagen Peptides in Human Blood Plasma after Ingestion of Collagen Hydrolysate and Cooked Shark Tail Meat.

	samples	AUC (μmol/h/L)	rate	*p*-value
Pro-Hyp	shark	1415.4 ± 203.2	0.34	0.001
	hydrolysate	4743.0 ± 701.8		
Hyp-Gly	shark	482.9 ± 142.7	0.73	0.725
	hydrolysate	554.1 ± 135.6		
Ala-Hyp	shark	77.6 ± 11.4	0.26	0.001
	hydrolysate	297.6 ± 47.2		
Ala-Hyp-Gly	shark	82.1 ± 21.7	0.29	0.028
	hydrolysate	281.5 ± 75.1		
Pro-Hyp-Gly	shark	92.5 ± 35.9	0.51	0.294
	hydrolysate	183.0 ± 73.6		
Leu-Hyp	shark	65.9 ± 5.5	0.38	0.013
	hydrolysate	171.4 ± 23.4		
Phe-Hyp	shark	40.7 ± 7.0	0.28	0.003
	hydrolysate	145.2 ± 25.4		
Ser-Hyp-Gly	shark	22.6 ± 4.5	0.17	0.001
	hydrolysate	134.2 ± 16.4		
Ile-Hyp	shark	24.5 ± 6.2	0.69	0.245
	hydrolysate	35.6 ± 6.5		
Free Hyp	shark	11390 ± 1720	0.61	0.0004
	hydrolysate	18774 ± 1927		
Hyp-containing peptide	shark	3571 ± 2298	0.28	0.0002
	hydrolysate	13012 ± 3599		

Values are mean ± S.E. (n = 6).

In Vitro Extraction and Enzyme Digestion of the Collagen in Shark Meat

To address the reason for the lower collagen peptide contents in plasma after ingestion of cooked shark meat compared to the contents after ingestion of collagen hydrolysate, the collagen in raw and cooked shark meat was extracted with hot water and digested with pepsin and pancreatin. The collagen hydrolysate is water soluble. In contrast, only ~10% of the total Hyp-containing protein/peptide was extracted from the raw and cooked shark meat by hot water at 100 °C (Figure 1-4). An additional 20% of the total Hyp-containing peptides were liberated by in vitro digestion with pepsin and pancreatin (Figure 1-4). The triple-helical collagen molecules are generally covalently cross-linked in tissue, which makes then water insoluble. Heat treatment with water cleaves the heat-labile crosslinks and converts the triple helical structure to a water-soluble globular structure.[3] Thus, heat treatment solubilizes a portion of the collagen and converts it to gelatin, which can be degraded by endoproteinases. However, collagen molecules stabilized with many heat-stable crosslinks are resistant to protease digestion, even after heat treatment.[44] The degree and types of crosslinks in collagen depend on the species and tissue.[3,45] These facts indicate that the collagen in the shark meat is stabilized

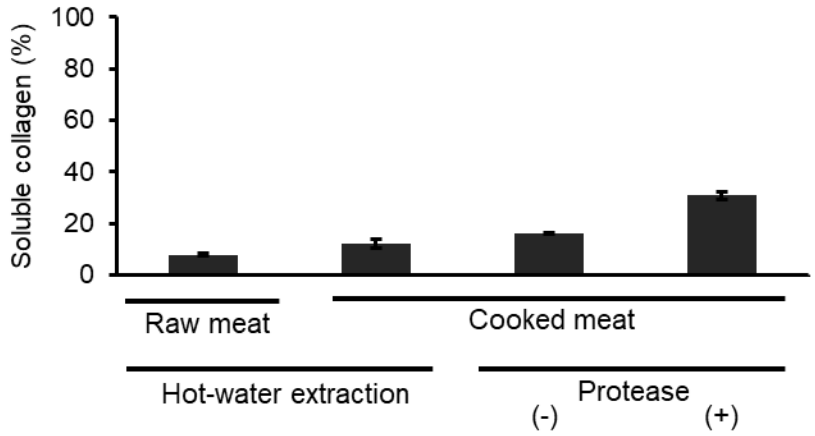

Figure 1-4. Extraction of collagen from raw and cooked shark tail meat (part D) by hot-water treatment and pepsin-pancreatin digestion. Data are mean ± S.D. (n = 3).

with crosslinks. Therefore, approximately 70% of the collagen content remained insoluble after cooking and pepsin-pancreatin digestion, which is consistent with the lower collagen peptide contents in plasma observed after ingestion of cooked shark meat compared to the contents after ingestion of collagen hydrolysate (Figure 1-4). Leucine aminopeptidase and carboxyl peptidase A degraded the peptides in the collagen hydrolysate with a molecular weight larger than 3000 Da into oligo peptides, which eluted in SEC after 25 min (Figure 1-5A and B). The exopeptidase digest of the peptides liberated from the cooked shark meat by pepsin-pancreatin digestion showed the SEC elution profile (Figure 1-5C) similar to that of the collagen hydrolysate (Figure 1-5B), which indicates that soluble collagen peptides from the cooked shark meat are further degraded by exopeptidases. Therefore, it is critical to increase the susceptibility of the collagen in meat to the gastrointestinal endoproteinases to generate soluble peptides. Collagen peptides might be also generated by autolysis in meat after long-term aging process and in marinade meat by acidic proteases such as cathepsins D and L,[46] while type I collagen, major collagen in fish meats, is stable during short-term chilled storage.[47] In addition, hot water extraction at a higher temperature (120 °C) in an autoclave solubilizes all the collagen in fish meat.[29] Thus, there is a possibility that cooking meat at a higher temperature in can and retort pouch for a longer time might increase the collagen peptide contents after ingestion of the cooked meat.

Consequently, approximately 0.2–13 g of collagen is ingested from the meat contained in one serving of a Japanese meal. Ingestion of this cooked meat can increase the di- and tri-collagen peptide contents in plasma, although the content is lower than that after ingestion of an equivalent amount of collagen hydrolysate, which suggests that collagen in daily dishes might affect on skin and joint conditions through increasing Pro-Hyp and Hyp-Gly in human blood. The collagen peptide contents in human blood after ingestion of meat not only depend on the collagen content in meat but also the degree of crosslinking and its susceptibility to endoproteinase digestion. The present study

encourages further human studies on the effects of ingesting soluble collagen in cooked meat on skin and joint conditions.

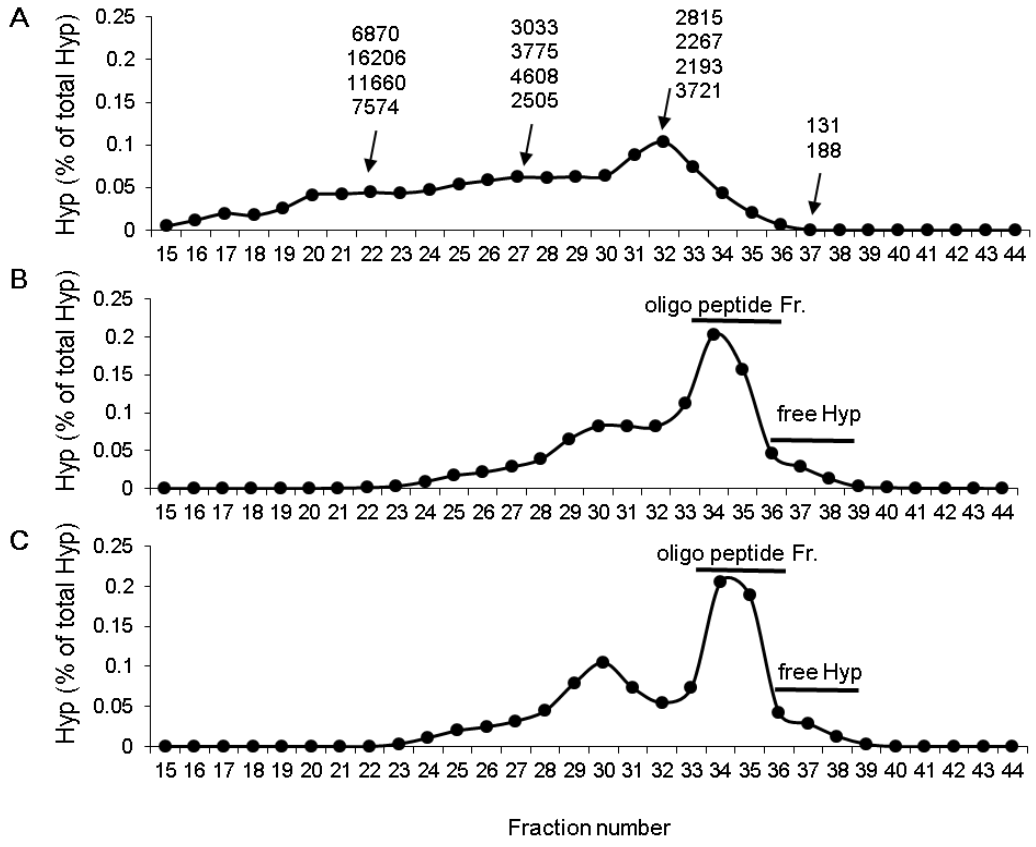

Figure 1-5. Elution profiles of collagen peptides from size exclusion chromatography. A, collagen hydrolysate; B, exopeptidase digest of collagen hydrolysate; and C, exopeptidase digest of collagen peptides in a pepsin-pancreatin digest of cooked shark meat. Elution of collagen peptides was monitored by measuring the Hyp contents in the HCl hydrolysate. The values at the arrows indicate the molecular weights of the collagen peptides as estimated by LC-MS.

Chapter 2

Food-derived collagen peptides, prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly), enhance growth of primary cultured mouse skin fibroblast using fetal bovine serum free from hydroxyprolyl peptide

2.1 Introduction

Collagen is the main protein in extracellular matrix and forms a triple-helical structure. Collagen has two specific post-translationally modified amino acids: hydroxyproline (Hyp) and hydroxylysine (Hyl). Heat treatment converts the triple-helical structure of collagen to a globular structure, which is referred to as gelatin. The protease digest of gelatin is referred to as collagen hydrolysate or collagen peptide. In human trials with placebo controls, ingestion of collagen hydrolysate moderates the symptoms of osteoarthritis[9,10] and enhances healing of pressure ulcers.[7,8,48] Furthermore, ingestion of collagen hydrolysate (2–20 g) or cooked meat increases the appearance of the peptide forms of Hyp (i. e. hydroxyprolyl peptide) in human peripheral blood plasma to 20–200 μM.[13–17] The presence of more than ten food-derived hydroxyprolyl peptides in human blood has been reported.[13–17] Pro-Hyp and Hyp-Gly are the main hydroxyprolyl peptides found in human blood after ingestion of collagen hydrolysate. Pro-Hyp is also generated by the degradation of endogenous collagen in tissue undergoing inflammation[21] and at wound healing sites in the skin.[22]

Mouse skin fibroblasts attached to collagen gel stopped growing without addition of Pro-Hyp even in the presence of fetal bovine serum (FBS), whereas fibroblasts grew on

plastic plates in the presence of FBS.[18,19,49] It has been reported that Pro-Hyp and Hyp-Gly triggered the growth of fibroblasts attached to collagen gel,[18,19] which has been associated with biological responses upon ingestion of collagen hydrolysate. However, some researchers have obtained results inconsistent with these findings (personal communication). In this chapter, it was found out that fibroblasts attached to collagen gel grew without adding Pro-Hyp, when using different lots of FBS than those used in previous studies. The objectives of chapter 2 were to solve this problem and confirm the effects of food-derived hydroxyprolyl peptides on the growth of fibroblasts.

2.2 Materials and Methods

Bovine Serums

Three different brands of FBS (FBS-1–3) were commercially obtained. One brand of adult bovine serum (ABS) was commercially obtained.

Chemicals

The amino acid standard mixture (Type H), acetonitrile (HPLC-grade), trimethylamine, and phenyl isothiocyanate were all purchased from Wako Chemicals (Osaka, Japan). Hydroxyproline and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution were purchased from Nacalai Tesque (Kyoto, Japan). Pro-Hyp and Hyp-Gly were obtained from Bachem (Bubendorf, Switzerland). Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (584 mg/L) was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s phosphate-buffered saline (D-PBS) and gentamicin were purchased from Invitrogen (Carlsbad, CA). Calf acid-soluble type I collagen solution (0.5%) was purchased from Koken (Tokyo, Japan). Cell Counting Kit-

8 was purchased from Dojin Glocal (Kumamoto, Japan). 6-Aminoquinolyl-N-hydroxy succinimidyl carbamate (AccQ) was obtained from Waters Corporation (Milford, MA). All other reagents were of analytical grade or better.

Removal of Hydroxyprolyl Peptides from FBS-1

An Econo-Pac 10DG column (Bio-Rad Laboratories, Hercules, CA) was pre-equilibrated with the DMEM. Three mL of FBS-1 was loaded onto the column. After elution of the first 3 mL, 13 mL DMEM were loaded onto the column. Every 1 mL of effluent was collected. Elution of protein was monitored by the Bradford method using a Protein Assay Kit (Bio-Rad Laboratories). Hydroxyproline and hydroxyprolyl peptides were detected by amino acid analysis as described previously in chapter 1.

Animals

All experiments were performed according to the ethical guidelines of the Kyoto University Animal Research Committee. The protocol was approved by the Kyoto University Animal Research Committee (permission number: 2014–45, 2015–38). All efforts were made to minimize the number of animals used and limit experimentation to only what was necessary. Five-week-old male Balb/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were sacrificed by cervical dislocation under deep isoflurane anesthesia. Abdominal skin was sterilized with 70% ethanol, shaved using a razor, and then stripped for use in these experiments.

Estimation of the Number of Cells Migrated from Mouse Skin

The skin was rinsed with D-PBS and DMEM to remove ethanol and placed on sterilized rubber plates. Disks measuring 4 mm in diameter were punched out using a Dermal Punch (Nipro, Tokyo, Japan). The skin disks were then placed on 12-well plastic plates (Falcon BD, Lakes, NJ). DMEM was supplemented with gentamicin (0.01 mg/mL),

FBS-1 (0%, 2%, and 10%) free from LMW hydroxyprolyl peptides, and Pro-Hyp (0 and 200 μM), respectively. One mL of each mixture was added to the wells. The 12-well plastic plates were placed in a humidified incubator at 37 °C under 5% CO_2. After incubation at suitable intervals, cells were fixed with 4% paraformaldehyde and observed using a phase-contrast microscope. The number of cells attached to the plate was directly counted.

Cell Proliferation Assay

Mouse abdominal skin was cut into square pieces (approximately 6–7 mm in width). Five pieces were placed on a culture dish (90 mm i.d.). Cultivation was carried out in 5 mL DMEM supplemented with gentamicin (0.01 mg/mL) and 10% FBS-1 or FBS-1 free from LMW hydroxyprolyl peptides in a humidified incubator at 37 °C under 5% CO_2. During cultivation, the medium was changed every 2–3 days. After incubation for 2 weeks, the skin disks were removed; the fibroblasts migrated from skin were washed with PBS and treated with 1 mL of a 0.25% trypsin-EDTA solution at 37 °C for 10 min. Nine mL of medium with 10% FBS-1 was poured into the plate to inactivate trypsin. Fibroblasts were collected by centrifugation at 3,000 × g for 10 min. The pellets were suspended in each medium; DMEM only, medium containing FBS-1, FBS-1 free from LMW hydroxyprolyl peptides, or LMW fraction of FBS-1 (5×10^4 cells/mL), with or without the addition of Pro-Hyp (final concentration 100 μM) and Hyp-Gly (100 μM). Fibroblasts (5×10^3 cells/100 μL) were cultured on 96-well plastic plates or collagen gel-coated plates in each medium; DMEM only, medium containing FBS-1, FBS-1 free from LMW hydroxyprolyl peptides, or LMW fraction of FBS-1, with and without the addition of Pro-Hyp and Hyp-Gly. The collagen solution (0.5%) was mixed with the same volume of double concentrated DMEM medium. The mixture (100 μL) was poured into each well of the 96-well plastic plate, and the plate was then placed in a humidified incubator for 1

h at 37 °C under 5% CO_2 to allow gelation. Cell proliferation was monitored using a Cell Counting Kit-8.

Amino Acid Analysis

FBS, ABS, and size-exclusion chromatography (SEC) fractions of FBS-1 were mixed with three volumes of ethanol and centrifuged at 1,000 × *g* for 10 min. The supernatant was used as the 75% ethanol-soluble fraction. Next, 100 μL of the 75% ethanol-soluble fraction was dried in a glass tube (5 × 60 mm) and hydrolyzed by 6 M HCl vapor at 150 °C for 1 h, as described previously in chapter 1. The Hyp contents in the non-hydrolysate and HCl hydrolysate were determined according to the method of Bidlingmeyer et al.[35] with slight modifications.[13]

Determination of Pro-Hyp and Hyp-Gly

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed in the multiple reaction monitoring (MRM) mode, using an LCMS-8040 (Shimadzu, Kyoto, Japan) and high pressure binary gradient HPLC (LC20 system, Shimadzu).

The 75% ethanol-soluble fraction (100 μL) was dried in a 1.5 mL tube and combined with 20 μL of 20 mM HCl, 20 μL of a 0.3% AccQ acetonitrile solution, and 60 μL of 50 mM sodium borate buffer (pH 8.8). The reaction mixture was kept at 50°C for 20 min and then diluted with 100 μL of 5 mM sodium phosphate buffer (pH 7.4) containing 10% acetonitrile. Authentic Pro-Hyp and Hyp-Gly were used for optimization of MRM conditions using LabSolutions Version 5.65 (Shimadzu). Aliquots (10 μL) were injected into the LC-MS/MS system. AccQ peptides were resolved on an Inertsil ODS-3 column (2.5 μm, 2.1 × 250 mm; GL Science, Tokyo, Japan) equilibrated with 0.1% formic acid (solvent A). Binary gradient elution was applied with solvent A and solvent B (0.1% formic acid containing 80% acetonitrile) at a flow rate of 0.2 mL/min. The gradient profiles were as follows: 0–15 min, 0%–50% B; 15–20 min, 50%–100% B; 20–25 min,

100% B; and 25–35 min, 0% B. The sample was spiked with standard peptides (5 pmol/injection) and recoveries of the spiked standards were calculated. To correct the matrix effect on ionization of sample peptides, the value of each peptide was divided by the recovery of each standard added to the sample.

Statistical analysis

Differences between means were evaluated using one-way analysis of variance, followed by Tukey's multiple comparison test for post hoc analysis using GraphPad Prism Version 6.04 (GraphPad Software, San Diego, CA). Differences between the two groups were compared using Student's *t*-tests.

2.3 Results and Discussion

Growth of Fibroblasts on Collagen Gel in Medium Containing FBS-1

Mouse skin fibroblasts were cultivated on collagen gel in a medium containing a commercially available lot of FBS (FBS-1). As shown in Figure 2-1, fibroblasts grew on the collagen gel even without addition of Pro-Hyp and Hyp-Gly. Addition of Pro-Hyp (100 μM) and Hyp-Gly (100 μM) did not significantly enhance growth of fibroblasts. These results are inconsistent with previous studies using different lots of the same brand of FBS, in which fibroblasts grew on collagen gel only after adding Pro-Hyp and Hyp-Gly.[18,19,49] It has been demonstrated that some FBS lots contain significant amounts of free Hyp, while the presence of hydroxyprolyl peptide has not been examined.[50] It was assumed that FBS might contain different levels of hydroxyprolyl peptides depending on lot number and brand, which might explain the inconsistent results.

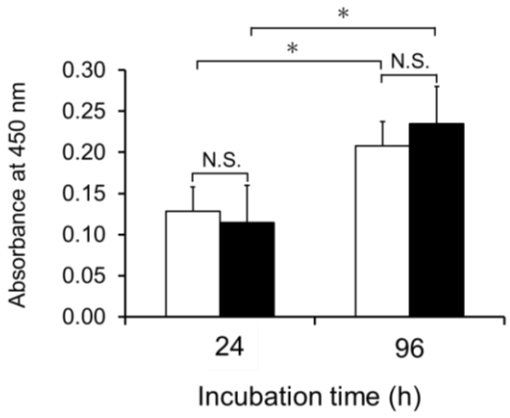

Figure 2-1. Effect of a mixture of Pro-Hyp and Hyp-Gly on the growth of fibroblasts on collagen gel in the presence of 10% FBS-1. (□), control; (■), medium containing Pro-Hyp and Hyp-Gly at 100 μM, respectively. Data are shown as mean ± S.D. (n = 5). Asterisks indicate significant differences ($p < 0.05$; Tukey's test). N.S. indicates results that are not significantly different.

Presence of Hydroxyprolyl Peptides in FBS

Amino acid analysis revealed presence of hydroxyprolyl peptides in the type of FBS used in the present study. As shown in Figure 2-2A, FBS contained unexpectedly higher levels of hydroxyprolyl peptide (approximately 70–100 μM) than adult bovine serum (ABS) and human plasma before ingestion of collagen hydrolysate.[13–17] As shown in Figure 2-2B, Pro-Hyp accounted for 37–70% of total hydroxyprolyl peptides in FBS. These values are similar to those in human plasma after ingestion of collagen hydrolysate.[13–17] On the other hand, only negligible amounts of Hyp-Gly were present in the FBS. Constituents in FBS differ between lots, even in the same brand.[50] Thus, different lots of FBS might contain different levels of Pro-Hyp and other hydroxyprolyl peptides.

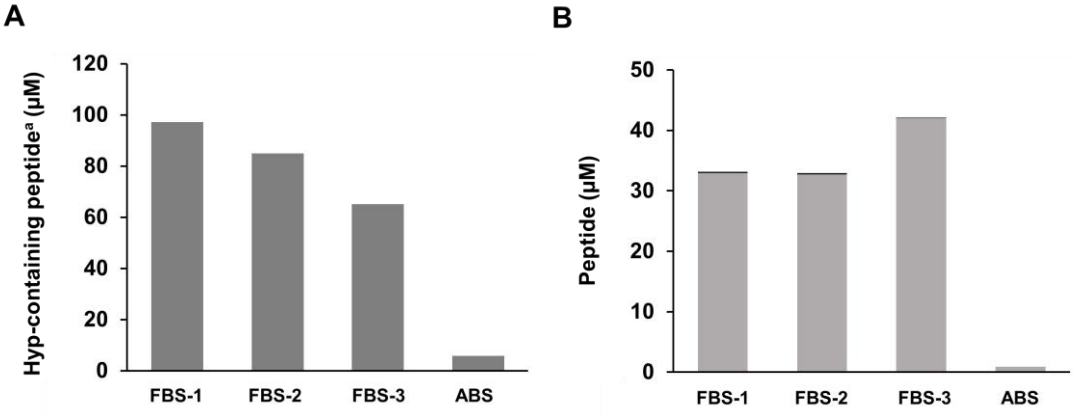

Figure 2-2. Contents of hydroxyprolyl peptide in commercially available fetal bovine serum (FBS) and adult bovine serum (ABS). A, Hyp-containing peptide; B, content of Pro-Hyp (■) and Hyp-Gly (■). [a]peptide form of Hyp.

Removal of Hydroxyprolyl Peptides from FBS-1

As shown in Figure 2-3, the protein in FBS-1 was eluted in fractions 4–7 by SEC. Hydroxyprolyl peptides were eluted in fractions 6–10. According to the instructions from the supplier, peptides larger than 6000 Da are eluted in fractions 4–7. Thus, hydroxyprolyl peptides in fractions 6 and 7 were larger than 6000 Da. Based on these facts, fractions 4–7 were collected and used as FBS-1 free from low molecular weight (LMW) compounds. Fraction 8–13 was used as LMW fraction.

The different fractions (FBS-1, FBS-1 free from LMW fraction, and LMW fraction) were each added to the medium of fibroblasts attached to plastic plates. FBS-1 free from LMW fraction caused fibroblast proliferation equivalent to proliferation with non-purified FBS-1 (Figure 2-4), while LMW fraction caused little fibroblast proliferation. These facts indicate that protein growth factors play a significant role in proliferating fibroblasts on plastic plates, as compared to LMW compounds in FBS.

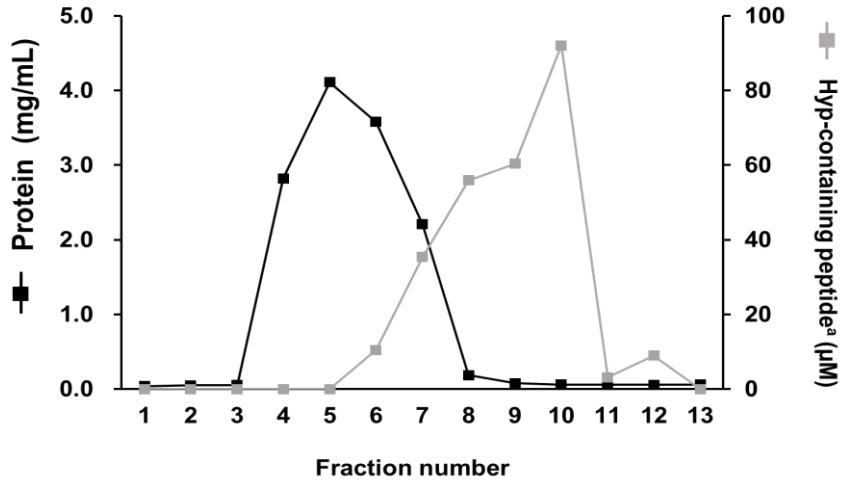

Figure 2-3. Elution of protein and hydroxyprolyl peptide in FBS-1 from the Econo-Pac 10DG column. (■), protein; (■), Hyp-containing peptide. [a]peptide form of Hyp.

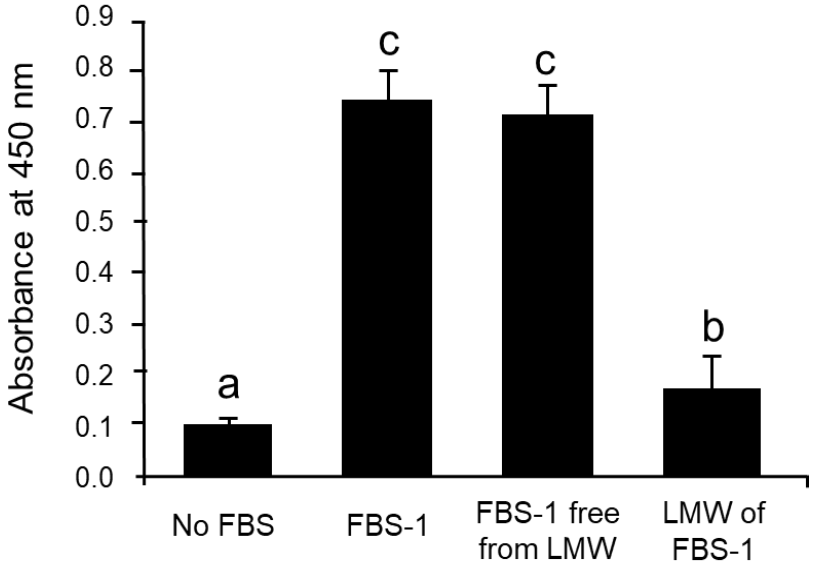

Figure 2-4. Effect of FBS-1 and its fractions on fibroblast growth on plastic plates. FBS-1 free from LMW, SEC Fr. 4–7; LMW of FBS-1, SEC Fr.8–13. Data are shown as the mean ± S.D. (n = 5). Different letters indicate significant differences ($p < 0.05$, Tukey's test).

Effect of Hydroxyprolyl Peptides on Growth of Fibroblasts on Collagen Gel

As shown Figure 2-5, fibroblasts attached to collagen gel stopped growing in the presence of FBS-1 free from LMW compounds. Addition of Pro-Hyp (100 μM) and Hyp-Gly (100 μM) triggered growth of the fibroblasts. These results are consistent to our previous studies.[1918] However, when the present FBS-1 was used without purification, entirely different results were obtained (Figure 2-1), due to presence of hydroxyprolyl peptides in the FBS-1.

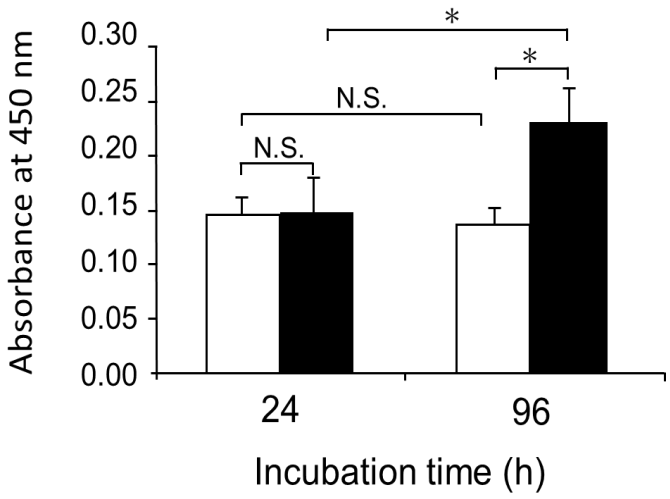

Figure 2-5. Effect of mixture of Pro-Hyp and Hyp-Gly on the growth of fibroblasts on collagen gel in the presence of 10% FBS-1 free from LMW hydroxyprolyl peptides. (□), control; (■), medium containing Pro-Hyp and Hyp-Gly at 100 μM, respectively. Data are shown as mean ± S.D. (n = 5). Asterisks indicate significant differences ($p < 0.05$, Tukey's test). N.S. indicates results that are not significantly different.

Effect of Pro-Hyp on Number of Fibroblasts Migrated from Mouse Skin

In previous research, it has been demonstrated that Pro-Hyp (200 μM) increased the number of fibroblasts that migrated from skin in the absence of FBS.[19] However, the addition of FBS removed this effect.[19] The FBS-1 free from LMW compounds was added to the skin culture system to give 2% and 10%. As shown in Figure 2-6, the number of fibroblasts that migrated from skin was significantly increased by the addition of Pro-Hyp (200 μM) in absence of FBS after 72 h, which is consistent with previous results.[19] Even in the presence of FBS-1 free from LMW compounds at 2% and 10%, Pro-Hyp also increased the number of fibroblasts migrating from skin after 48 and 24 h, respectively.

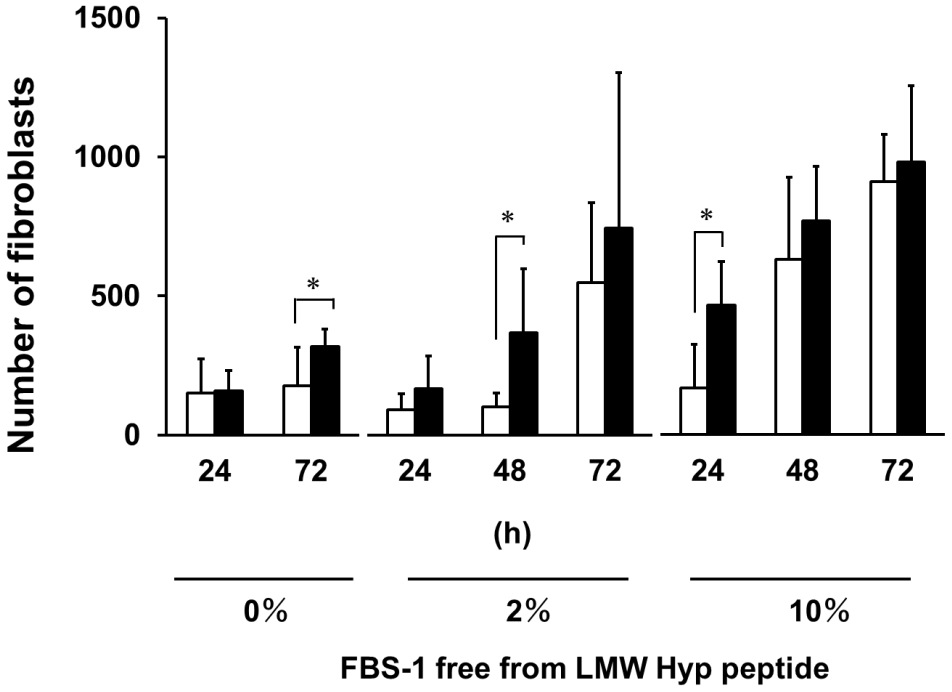

Figure 2-6. Effect of Pro-Hyp on the number of fibroblasts migrated from mouse skin in the absence or presence of FBS-1 free from LMW hydroxyprolyl peptides. Open column, control; closed column, medium containing Pro-Hyp at 200 μM. Data are shown as the mean ± S.D. (n = 6). Asterisks indicate significant differences ($p < 0.05$, Student's *t*-test).

In the previous study,[19] hydroxyprolyl peptides in FBS might have partially masked the effect of Pro-Hyp on the number of fibroblasts migrating from mouse skin.

2.4 Conclusions

The present study demonstrates that some commercially available FBS sera contain high levels of LMW hydroxyprolyl peptides (70-100 μM) including Pro-Hyp. These values are higher than those in ABS and human plasma without ingestion of collagen hydrolysate. By using FBS that is free from LMW hydroxyprolyl peptides, the present study clearly confirms that Pro-Hyp and Hyp-Gly play crucial roles in proliferation of fibroblasts attached to collagen gel.

Chapter 3

Mouse skin fibroblasts with mesenchymal stem cell marker p75 neurotrophin receptor proliferate in response to prolyl-hydroxyproline

3.1 Introduction

Collagen is a primary protein component of the extracellular matrix and has specific amino acids such as hydroxyproline (Hyp) and hydroxylysine. Heat-denatured collagen is known to gelatin and the enzymatic hydrolysate of gelatin is used as a food ingredient and is referred to as collagen hydrolysate. Human trials have demonstrated that ingestion of collagen hydrolysate has beneficial effects on human health and increase blood levels of some collagen-derived di- and tri-peptides such as prolyl-hydroxyproline (Pro-Hyp), hydroxyprolyl-glycine (Hyp-Gly), and Pro-Hyp-Gly in humans.[13–17] The chapter 1 has demonstrated that ingestion of gelatin also increase the levels of collagen peptides in human blood. Pro-Hyp is also generated in mouse ears affected with dermatitis and at sites of skin wound healing.[21,22] Pro-Hyp and Hyp-Gly have been demonstrated to trigger the growth of primary cultured mouse skin fibroblasts attached on collagen gel.[18,19] This suggests that Pro-Hyp plays an important role in wound healing by enhancing the growth of fibroblasts, which are vital to this process.

On the other hand, these findings also suggest that food-derived Pro-Hyp might enhance growth of resident fibroblasts in normal tissues and induce lesions. However, it has not been reported that ingestion of collagen hydrolysate induces abnormal proliferation of fibroblasts or other pathological changes in the body, even following long-term administration.[7–9] In addition, it has been reported that some established fibroblast

cell lines do not respond to Pro-Hyp.[19] This indicates the presence of both Pro-Hyp responding and non-responding fibroblasts in the body.

Nerve growth factor (neurotrophin) low-affinity receptor—known as p75 neurotrophin receptor (p75NTR), p75 nerve growth factor receptor (NGFR), low-affinity NGFR, or CD271 was initially discovered in neuronal cells.[51] It is now known that p75NTR is also a mesenchymal stem cell marker.[52–54] In addition, recent studies have shown that p75NTR is expressed in various non-neuronal cell types, such as fibroblasts and macrophages under inflammatory conditions.[55–58] These studies indicate that p75NTR-positive cells play an important role in wound healing.

The aim of the present study was to examine the relationship between expression of p75NTR and response to Pro-Hyp using primary cultured mouse skin fibroblasts. The results showed that Pro-Hyp specifically enhances growth of p75NTR-postive fibroblasts.

3.2 Materials and Methods

Chemicals

Dulbecco's phosphate-buffered saline (D-PBS), Dulbecco's Modified Eagle's Medium (DMEM) (1.0 g/L glucose), penicillin–streptomycin solution, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, L-hydroxyproline (Hyp), isoflurane, and fluorescein isothiocyanate (FITC) isomer were purchased from Nacalai Tesque (Kyoto, Japan). Pro-Hyp was obtained from Bachem (Budendorf, Switzerland). DMEM-low glucose powder, gelatin from cold water fish skin, bovine serum albumin (BSA), goat serum, 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride, and paraformaldehyde (PFA) were obtained from Sigma-Aldrich (St. Louis, MO). Calf acid-soluble type I collagen solution (0.5%) was purchased from Koken (Tokyo, Japan). Gentamicin was purchased from Gibco (Carlsbad, CA). Cell Counting Kit-8 was purchased from Dojin

Glocal (Kumamoto, Japan). 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemicals (Ontario, Canada). Fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France). We used an Econo-Pac 10DG column (Bio-Rad Laboratories, Hercules, CA) to remove any hydroxyprolyl peptides, such as Pro-Hyp, from the FBS. FBS (3 mL) was loaded onto a column pre-equilibrated with DMEM. After elution of the first 3 mL, DMEM (4 mL) was loaded onto the column. The effluent was collected and used as FBS free from hydroxyprolyl peptides (FBS-FHP). All other reagents were of analytical grade or better.

Preparation of FITC-labeled Pro-Hyp

Pro-Hyp (14 μmol) was dissolved in 1 mL of 0.1 M Na_2CO_3 buffer (pH 7.5). One hundred μL of 0.02% (w/v) FITC solution in water was added to 900 μL of the Pro-Hyp solution. The mixture was shielded with aluminum foil and kept at 4°C overnight. FITC-labeled Pro-Hyp was purified via reversed phase high performance liquid chromatography (HPLC) using a Cosmosil 5C18-MS-II column (10 mm i.d. × 250 mm, Nacalai Tesque). A binary linear gradient was used with 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at a flow rate of 2 mL/min. The gradient profile was as follows: 0–20 min, 0%–50% B; 20–30 min, 50%–100% B; 30–35 min, 100% B; and 35–35.1 min, 100–0% B; 35.1–45 min, 0% B. The column was maintained at 40°C. Elution of peptide was monitored at 214 and 254 nm. Main peaks were collected. FITC-labeled Pro-Hyp was detected by electron spray ionization mass spectrometry using an LCMS-8040 (Shimadzu, Kyoto, Japan) in flow injection mode.

Cell Culture

Fibroblasts were collected from mouse skin. Five-week-old male Balb/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were sacrificed by cervical dislocation under deep isoflurane anesthesia and skin was sterilized with 70% ethanol.

The abdominal skin was shaved with a razor and immediately dissected away using scissors. The skin was rinsed with D-PBS to remove residual ethanol and was cut into square pieces (~6–7 mm in width). Five pieces were placed directly on polystyrene culture dish (90 mm i.d.) and covered with 5 mL DMEM containing 10 μg/mL gentamicin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% FBS-FHP, and kept in a humidified incubator at 37°C under 5% CO_2. The medium was changed every 2–3 days without moving the skin.

To observe morphological changes of cells migrating from the skin, time-lapse photography of living cells was obtained using an All-in-One Fluorescence Microscope BZ-X710 (Keyence, Osaka, Japan). After 3 days of incubation, cells that had migrated from the skin were photographed every 1 min for 15 h.

For immunostaining of cells that had migrated from the skin, skin pieces and media were removed at suitable intervals. The cells were rinsed three times with D-PBS and fixed with 4% PFA in D-PBS for 15 min. The fixed cells were then rinsed three times with D-PBS and stained with antibodies as described below.

For observation of FITC-labeled Pro-Hyp incorporated into fibroblasts, skin pieces were removed at suitable intervals. Cells were then incubated in media containing FITC-labeled Pro-Hyp (10 μM) for 1 day, rinsed three times with D-PBS, and fixed with 4% paraformaldehyde, as described above.

Incorporation of Pro-Hyp into fibroblasts was directly examined by liquid chromatography tandem mass spectrometry (LC-MS/MS). After incubation of the mouse skin for 2 weeks, the skin pieces were removed. The fibroblasts outside of the skin were then incubated in media containing 200 μM Pro-Hyp for 3 days. Fibroblasts were washed three times with D-PBS and detached using 1 mL of a 0.25% trypsin-EDTA at 37°C for a few minutes. To inactivate the trypsin, 9 mL DMEM containing 10% FBS (without Econopac 10DG treatment) was added to the culture dish. Cell numbers were determined using a cell counting chamber (Hirschmann, Eberstadt, Germany) before centrifugation

at 3,000 × *g* for 10 min. The pellet was homogenized with 70% ethanol using a BioMasher II (Nippi, Tokyo, Japan). The suspension was then centrifuged at 10,000 × *g* for 5 min. Some of the fibroblasts outside of the mouse skin after 2 weeks of incubation were collected, as described above, and inoculated on the same dish (25×10^4 cells/5 mL media) and incubated for another week in media without Pro-Hyp. Thereafter, the fibroblasts were incubated in the medium containing Pro-Hyp for 3 days and the fibroblasts were collected and treated with 70% ethanol, as described above. Pro-Hyp content in the 70% ethanol supernatants was determined by LC-MS/MS.

To examine the effects of Pro-Hyp on growth of fibroblasts, fibroblasts outside of the skin pieces were collected after 2 and 4 weeks of incubation, as described above. Cells were resuspended at 5×10^4 cells/mL in DMEM containing 10% FBS-FHP in the presence or absence of 200 μM Pro-Hyp. The fibroblasts were cultured on a 48-well polystyrene plates (BD Bioscience, Bedford, MA), with or without collagen gel. The collagen solution (0.5%) was mixed with the same volume of double-concentrated DMEM. The mixture (100 μL) was poured into each well of the 48-well plate. The plate was placed in the humidified incubator for 30 min at 37°C under 5% CO_2 to allow gelation. The collagen gel was sterilized by ultraviolet lamp on a clean bench for 15 min. The fibroblast suspension (100 μL, 5×10^3 cells) was inoculated in the wells without and with collagen gel. The plates were then placed in a humidified incubator at 37°C under 5% CO_2 for 96 h.

Cell Proliferation Assay

To estimate cell growth, 20 μL of tetrazolium solution (Cell Counting Kit-8) was added to each well. The absorbance of the medium (70 μL) at 450 nm was measured using a Microplate Reader (Model 680, Bio-Rad Laboratories) 2 h after addition of the tetrazolium.

Immunocytochemical Staining

The fixed cells on polystyrene dishes were treated with blocking solution (5% BSA, 2% fish gelatin, and 5% goat serum) for 1 h and subsequently treated with rabbit polyclonal antibody against p75NTR (AB1554, Merck Millipore, Darmstadt, Germany), rabbit polyclonal antibody against wide-spectrum cytokeratin (ab9377, Abcam, Cambridge, UK), or goat polyclonal antibody against perilipin-1 (ab60269, Abcam) at room temperature for 1 h. All primary antibody solutions were diluted in the blocking solution at 1:500. After removal of primary antibodies, the cells were rinsed three times with D-PBS and treated with the following secondary antibodies: goat anti-rabbit IgG H&L with Alexa Fluor 488 (ab150077, Abcam) or Alexa Fluor 568 (ab175471, Abcam), and rabbit anti-goat IgG H&L with Alexa Fluor 568 (ab175707, Abcam), at room temperature for 1 h. All secondary antibodies were diluted in the blocking solution at 1:2,000. After immunostaining, cells were also stained with DAPI diluted in distilled water at 1:10,000 at room temperature for 10 min.

The fibroblasts on collagen were also fixed with 4% PFA, and stained with rabbit polyclonal antibody against p75NTR and DAPI as described above. After staining, the collagen gel with cells was picked up from the well with forceps and placed upside down in the polystyrene dish for observation by fluorescence microscopy. Fluorescence was observed with an inverted microscope (IX73; Olympus, Tokyo, Japan) fitted with a fluorescence unit (U-HGLGPS, Olympus).

LC-MS/MS Analysis

Pro-Hyp was identified by LC-MS/MS in the multiple reaction monitoring (MRM) mode using Q-TRAP 3200 (AB SCIEX, Foster City, CA) and high pressure binary gradient HPLC (Prominence 20A, Shimadzu). Aliquots of the 70% ethanol extract (50 μL) of fibroblasts were dried in 1.5 mL tubes under vacuum and peptides in the extract were derivatized with AccQ as described previously in chapter 1. The AccQ derivative of

authentic Pro-Hyp was used for optimization of MRM conditions using Analyst software (AB Sciex). Aliquots (10 μL) were injected into the LC-MS/MS and AccQ peptides were resolved on an Inertsil ODS-3 column (2.5 μm, 2.1 × 250 mm; GL Science, Tokyo, Japan) equilibrated with 0.1% formic acid containing 5% acetonitrile (solvent A). A binary gradient elution was applied with solvent A and solvent B (0.1% formic acid containing 80% acetonitrile) at a flow rate of 0.2 mL/min. The gradient profiles were as follows: 0–12 min, 0%–50% B; 12–20 min, 50%–100% B; 20–24 min, 100% B; and 24.01–30 min, 0% B.

Statistical Analyses

Values are expressed as means ± standard deviation. Differences between the means were evaluated by one-way analysis of variance followed by Tukey's multiple comparison test for post hoc analysis using GraphPad Prism Version 6.04 (GraphPad Software, San Diego, CA). Differences between the two groups were compared using Student's *t*-test or Mann–Whitney *U*-test. P values less than 0.05 were considered significant.

3.3 Results

Morphology of Cells Migrated from Mouse Skin

After incubating mouse skin for 3 days, a variety of cells with different shapes had migrated from the skin. As shown in Figure 3-1A, some small spherical floating cells (~5 μm) were observed (in red circle). Time-laps observations revealed that these cells were crawling on the dish (data not shown), identifying them as leukocytes. Many adhesive cells were also observed (Figure 3-1A). These cells had bipolar and multi-polar spindle-like shapes, which are typical of fibroblasts. In addition, adhesive cells with spherical

shape were observed. Time-lapse observations revealed that the spindle-like cells temporarily became spherical before dividing into two cells (Figure 3-1B). Therefore, the adhesive cells with spindle-like and spherical shapes were all considered fibroblasts. Adhesive cells with shapes different from fibroblasts migrated from specific areas of the skin and formed a cobblestone-like pattern (Figure 3-1C). As shown in Figure 3-2A, cells in the cobblestone-like area displayed cytokeratin (green) or perilipin (red), indicating that the cobblestone-like areas consisted of keratinocytes and adipocytes, respectively. Larger cells (~50 μm) with a fried egg-like shape and ruffled membranes, typical of macrophages,[59] were not observed among the cells that migrated from the skin.

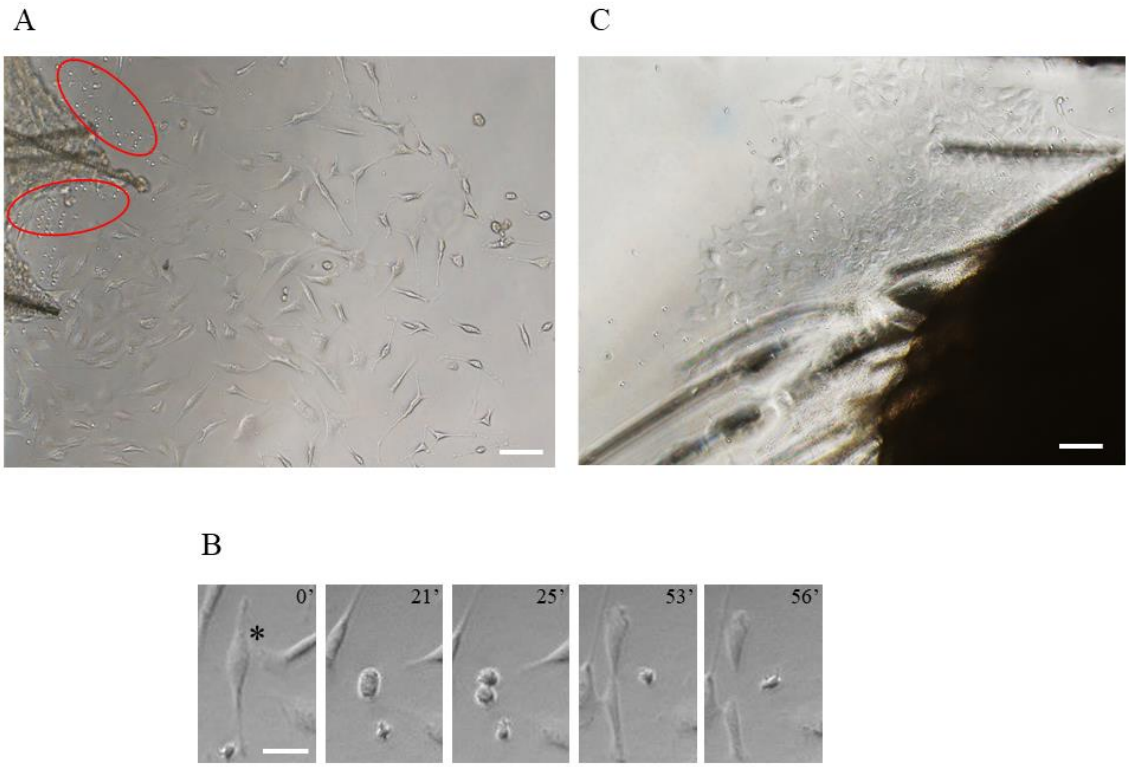

Figure 3-1. Morphology of cells outside of mouse skin after 3 days of incubation.

A, Representative picture of cells that had migrated from mouse skin. Spindle-like and spherical adhesive cells, as well as small floating spherical cells (in red circle), were observed. B, Time-lapse observation of spindle-like cells. A spindle-like cell (*) changed to a spherical morphology and divided into two cells. Time (min) after start of observation is inserted in each panel. C, Picture of cells that had migrated from different parts of the skin. Cells forming a cobblestone-like pattern. Black shadow is mouse skin with hair. Bars in panel A and C indicate 50 μm. Bar in panel B indicates 20 μm.

p75NTR-Positive and Negative Cells Migrated from Mouse Skin

Cells that had migrated from mouse skin after 1 day of incubation were immunostained with antibody against p75NTR. Keratinocytes (Figure 3-2B in red circle) did not express p75NTR, whereas, the fibroblasts with both spindle-like and spherical shapes did express p75NTR (Figure 3-2B). After 2 weeks of incubation, most of the

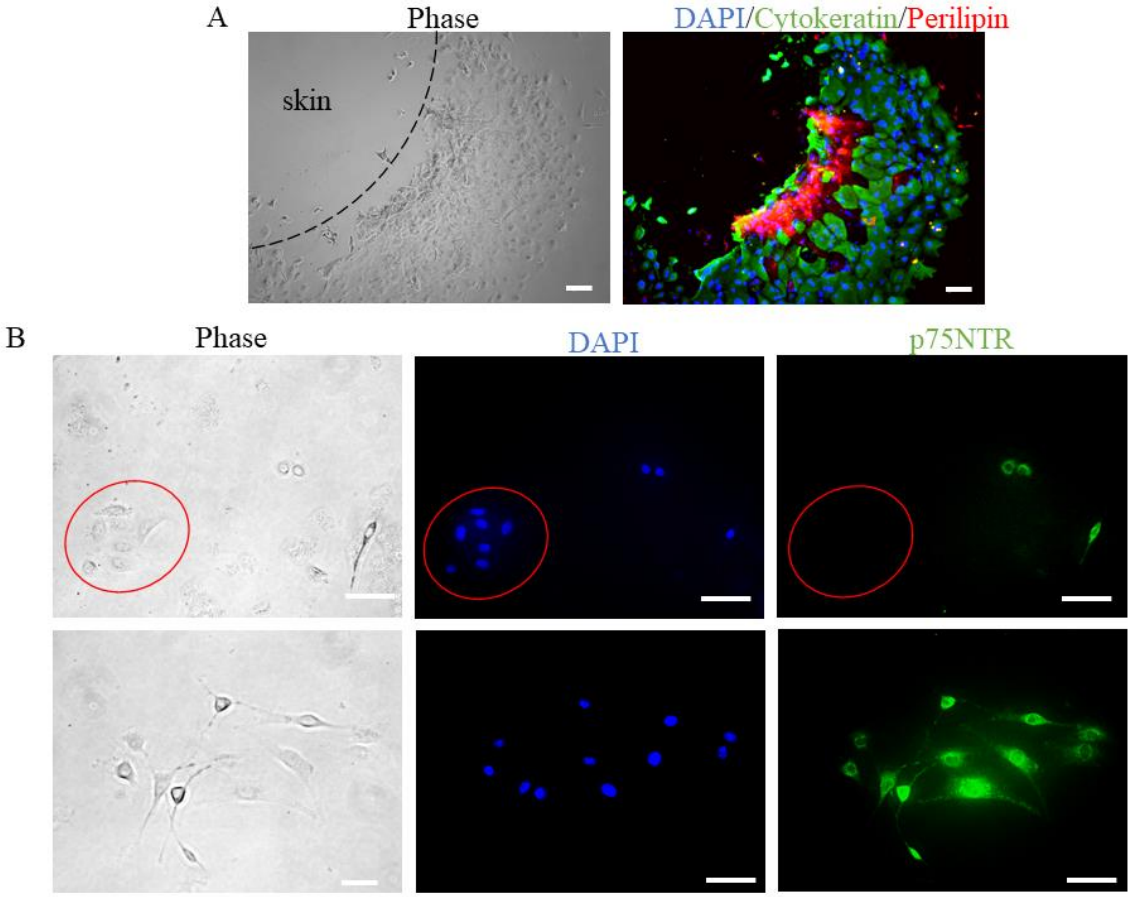

Figure 3-2. Immunocytochemistry of cells outside of mouse skin after 1 or 4 days of incubation. Cells were stained with DAPI (blue), antibodies against cytokeratin (green in panel A), p75NTR (green in panel B), and perilipin (red in panel A). A, Cells forming a cobblestone-like area after 4 days of incubation. B, Keratinocytes (in red circle) and fibroblasts with spindle-like and spherical shapes after 1 day of incubation. Bars in panel A and B indicate 100 and 50 μm, respectively.

fibroblasts outside of the skin had lost p75NTR expression, and only few fibroblasts near the skin still displayed p75NTR (Figure 3-3A). After 4 weeks of incubation, many fibroblasts were observed but they were completely devoid of p75NTR staining (Figure 3-3B).

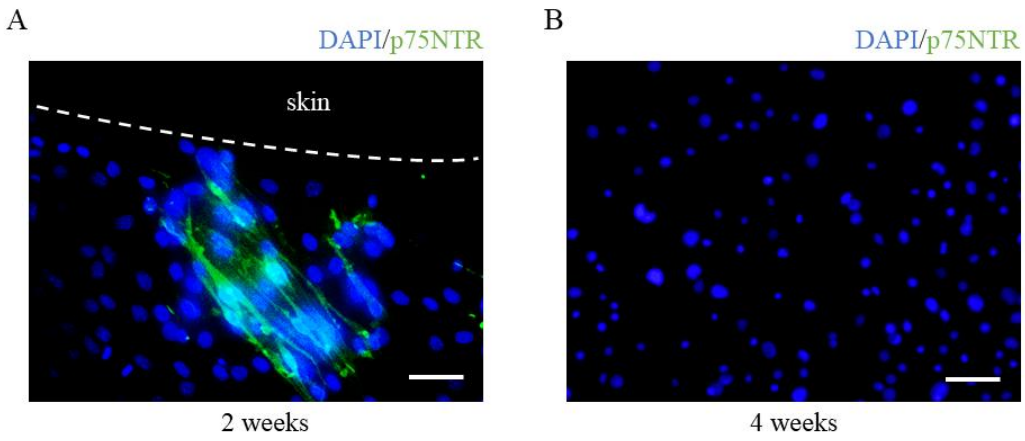

Figure 3-3. Immunocytochemistry of fibroblasts outside of mouse skin after 2 or 4 weeks of incubation. Cells were stained with DAPI (blue) and antibody against p75NTR (green). Bars indicate 50 μm.

Incorporation of Pro-Hyp in Fibroblasts

Relationship between expression of p75NTR and incorporation of Pro-Hyp was examined using FITC-labeled Pro-Hyp. All fibroblasts outside of the mouse skin after 1 day of incubation expressed p75NTR (red) and showed fluorescence of FITC-Pro-Hyp (green) (Figure 3-4A). After incubating the fibroblasts had lost p75NTR expression (Figure 3-4B). Green fluorescence of FITC Pro-Hyp was specifically observed in fibroblasts expressing p75NTR. The green fluorescence was strongly observed in the cytoplasm, while weaker fluorescence was observed in the nuclei (Figure 3-4 insert). This

indicates that FITC-labeled Pro-Hyp had moved into the cytoplasm rather than being bound to the cell surface.

LC-MS/MS analysis revealed that fibroblasts collected after 2 weeks of incubation incorporated Pro-Hyp in cells (Figure 3-5). However, incorporated Pro-Hyp levels were significantly decreased in fibroblasts collected after another week of incubation.

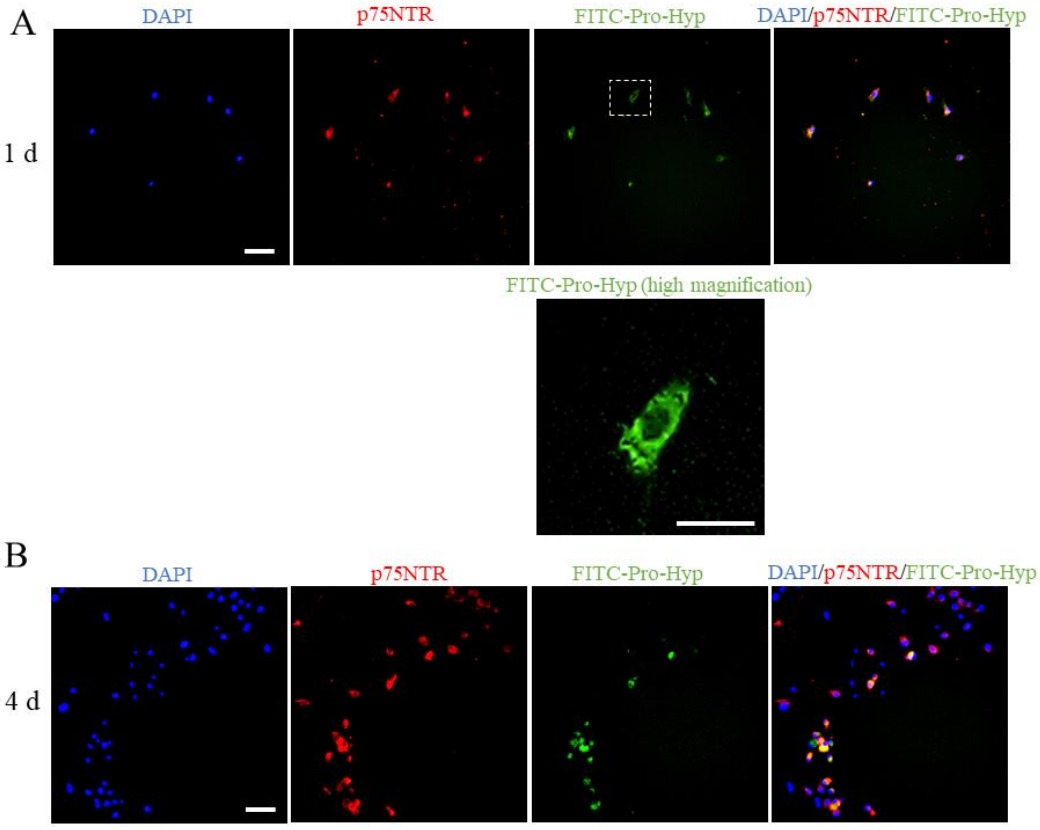

Figure 3-4. Incorporation of FITC-labeled Pro-Hyp into fibroblasts outside of the mouse skin. FITC-labeled Pro-Hyp was added to the media of fibroblasts outside of the skin after 1 (panels A) and 4 days (panels B) of incubation. After another day, fluorescence of FITC-labeled Pro-Hyp (green), DAPI (blue), and antibody against p75NTR (red) in fibroblasts were observed. Insert, high magnification picture of area indicated by solid line in panel A. Bars indicate 50 μm or 20 μm (high magnification).

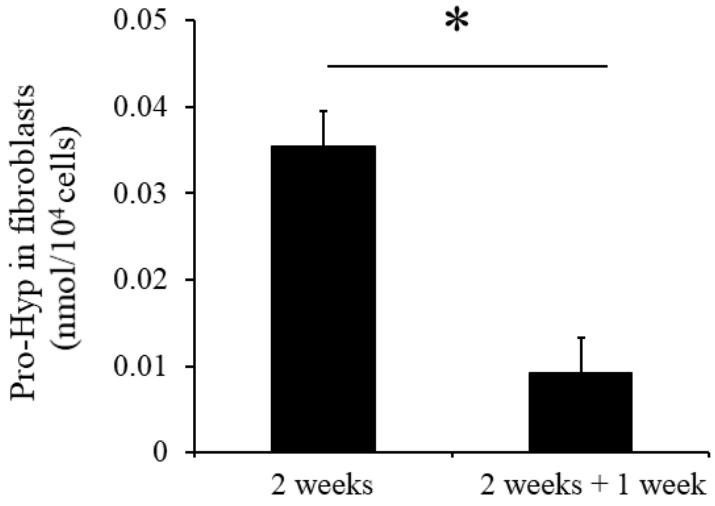

Figure 3-5. Determination of Pro-Hyp in fibroblasts by LC-MS/MS.
Fibroblasts outside of the skin after 2 weeks of incubation were collected (2 weeks). Some of the collected fibroblasts were further incubated for another week (2 weeks + 1 week). The collected fibroblasts were incubated in medium containing Pro-Hyp (200 μM) for 3 days. Pro-Hyp in fibroblasts was identified by LC-MS/MS. Mean ± S.D.; *$p < 0.05$; Student's *t*-test, n = 5.

Effect of Pro-Hyp on Growth of Fibroblasts

Fibroblasts outside of the skin were collected after 2 weeks of incubation and inoculated in wells without and with collagen gel. Pro-Hyp significantly enhanced the growth of fibroblasts not only on collagen gel, but also in uncoated wells (Figure 3-6). Fibroblasts on collagen gel were stained with antibody against p75NTR. However, it was difficult to detect fluorescence of the secondary antibody due to the high background fluorescence of the collagen (Figure 3-7A upper). Placing the collagen gel upside down enabled detection of fluorescence from the secondary antibody (Figure 3-7A lower). Treatment with Pro-Hyp significantly increased the ratio of p75NTR-positive fibroblasts

to total fibroblasts on the collagen gel (Figure 3-7B). On the other hand, fibroblasts that had completely lost p75NTR after 4 weeks of incubation did not respond to Pro-Hyp. This was true for fibroblasts either on collagen gel or in uncoated wells (Figure 3-8), though these cells still grew in the uncoated wells for 48–96 h.

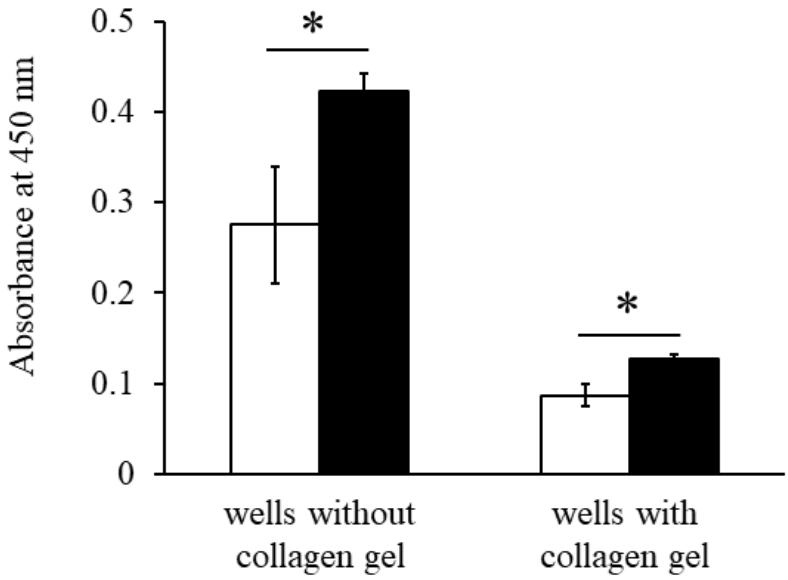

Figure 3-6. Effects of Pro-Hyp on growth of fibroblasts outside of the mouse skin after 2 weeks incubation. Fibroblasts were incubated in the wells without and with collagen gel. Open column, media only; closed column, media containing 200 μM Pro-Hyp. Mean ± S.D.; *, $p < 0.05$; Student's *t*-test, n = 6 wells.

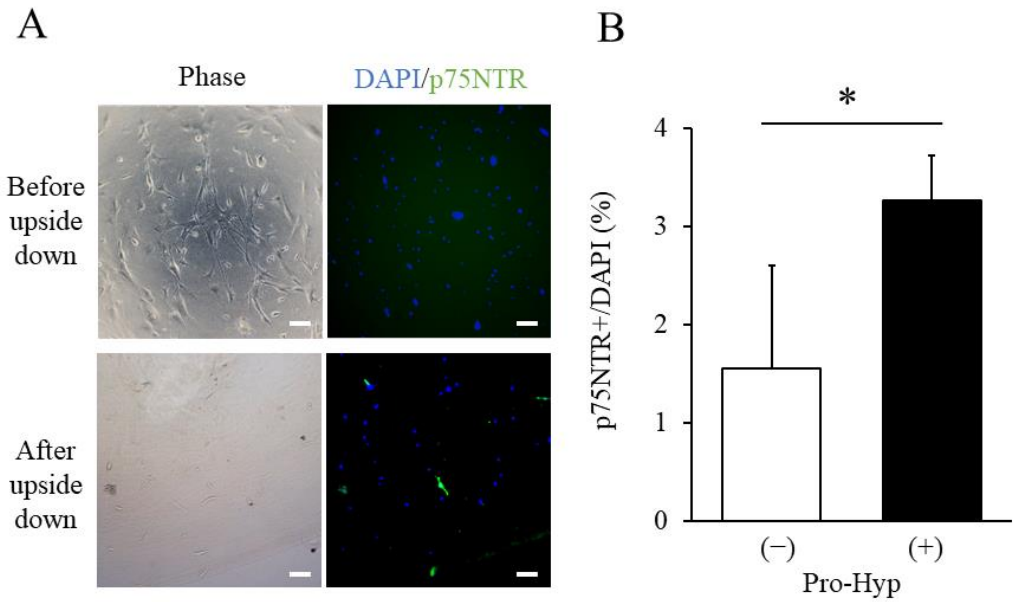

Figure 3-7. Effects of Pro-Hyp on the ratio of p75NTR-positive fibroblasts on collagen gel. Fibroblasts outside of the mouse skin after 2 weeks of incubation were collected and inoculated on collagen gel and incubated for 2 days in the presence or absence of 200 μM Pro-Hyp. Fibroblasts on collagen were observed directly (upper panels in A) and after putting the collagen gel upside down (lower panels in A) by inverted fluorescence microscopy. Bars indicate 50 μm. B, Ratio of p75NTR-positive fibroblasts. Open column, media only; closed column, media containing Pro-Hyp. Mean ± S.D.; *, $p < 0.05$; Mann‑Whitney *U*-test, n = 6 wells.

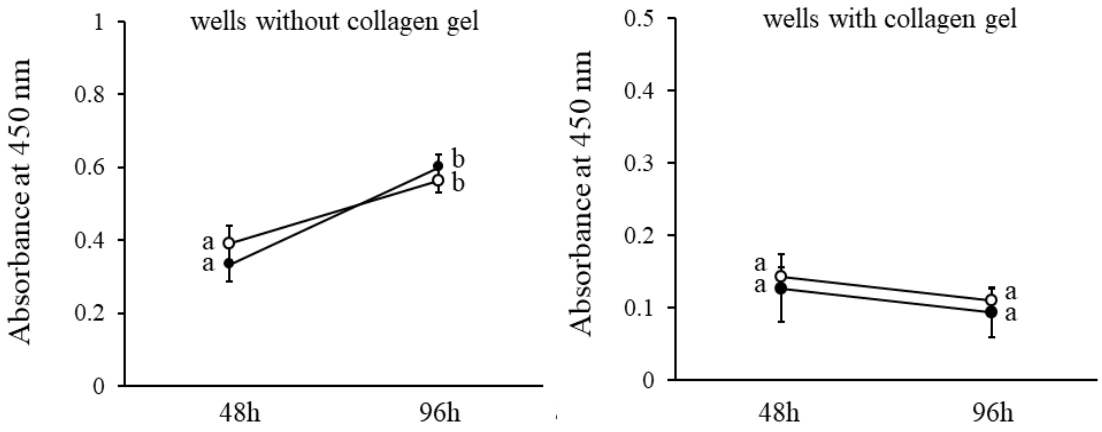

Figure 3-8. Effects of Pro-Hyp on growth of fibroblasts outside of mouse skin after 4 weeks incubation. Fibroblasts were incubated in wells without and with collagen gel. Open circle, media only; closed circle, media containing 200 μM Pro-Hyp. Mean ± S.D.; different letters, $p < 0.05$; Tuckey's test, n = 6 wells.

3.4 Discussion

The mouse skin culture system presented here has been used to evaluate the biological activity of Pro-Hyp.[18,19] In the previous studies, most of the cells that migrated outside of the mouse skin were considered fibroblasts, as these cells synthesized collagen and showed typical morphological feature of fibroblasts (i.e., spindle-like shapes).[18,19] It was demonstrated here that fibroblasts that migrated from the mouse skin temporally had spherical shapes as well as spindle-like shapes, and that leukocytes, keratinocytes, and adipocytes also migrated from the mouse skin (Figure 3-1, 3-2A). Fibroblasts outside of

the skin after several days of incubation, with both spindle-like and spherical shapes, expressed p75NTR, while keratinocytes did not express p75NTR (Figure 3-2B).

p75NTR is a known marker of mesenchymal stem cells, which can differentiate into osteoblasts, adipocytes, chondroblasts, and other cell types.[60,61] Palazzo et al demonstrated that primary cultured fibroblasts that migrated from human skin expressed p75NTR mRNA.[57] The present study revealed that the ratio of p75NTR-positive fibroblasts outside of the mouse skin decreased after prolonged incubation, while the number of overall fibroblasts increased (Figure 3-3). This suggests that the p75NTR-positive cells are fibroblasts newly differentiated from mesenchymal stem cells or mesenchymal stem cells with fibroblast-like shapes, which will subsequently change into fibroblasts. It is unlikely that some stimuli, such as growth factors in the FBS turn resident skin fibroblasts into p75NTR-positive fibroblasts, as the fraction of p75NTR-positive cells rapidly decreased even with the addition of fresh FBS.

It has been demonstrated that the fibroblasts that had grown outside of skin after 2 weeks of incubation stopped growing on collagen gel in the absence of Pro-Hyp, even in the presence of FBS.[19,49] Pro-Hyp triggered growth of the fibroblasts on collagen gel.[19] The present study demonstrates that the fibroblasts that had grown outside of skin after 2 weeks of incubation contained p75NTR-positive fibroblasts (Figure 3-3) and grew on collagen gel responding to Pro-Hyp (Figure 3-6), while the fibroblast that had grown outside of skin after prolonged incubation lost p75NTR (Figure 3-3) and did not grow on collagen gel in the presence of Pro-Hyp (Figure 3-8). In addition, Pro-Hyp increased the number and ratio of p75NTR-positive fibroblasts on collagen gel (Figure 3-6 and 3-7). Therefore, presence of p75NTR-positive fibroblasts is crucial for the Pro-Hyp-triggered growth on collagen gel.

Peptide transporters 1 and 2 (PepT1 and PepT2) have been shown to allow movement of FITC-labeled dipeptides into cells,[62] though it has been reported that human fibroblast cell lines do not express these transporters.[63] In our study, the fluorescence of

FITC-labeled Pro-Hyp was only observed in the cytoplasm of p75NTR-positive fibroblasts (Figure 3-4). LC-MS/MS analysis confirmed the presence of Pro-Hyp in fibroblasts that had grown outside of skin after 2 weeks of incubation, which included p75NTR-positive fibroblasts. These facts indicate that Pro-Hyp stimulates p75NTR-positive fibroblasts by incorporating into cytoplasm, while it does not affect p75NTR-negative fibroblasts.

It is well known that protein growth factors such as basic fibroblast growth factors and platelet-derived growth factor stimulate growth of fibroblasts.[64] Collagen dipeptides such as Pro-Hyp and Hyp-Gly also control fibroblast growth.[18,19] The present study demonstrates that expression of p75NTR and some peptide transporters, which incorporate Pro-Hyp into cells, also affects growth of fibroblasts. The number of cell divisions occurring after differentiation from mesenchymal stem cells may also affect sensitivity of the fibroblasts to collagen dipeptides, as p75NTR and peptide transporters disappeared after growth.

It has been demonstrated that p75NTR positive cells accumulate at sites of skin wound healing.[65] In vitro and in vivo studies have shown that p75NTR and neurotrophin play crucial roles in wound healing and tissue remodeling.[65–67] p75NTR is expressed in various cells types, including fibroblasts, particularly under inflammatory conditions.[55–58] Therefore, p75NTR-postive fibroblasts play a significant role in wound healing by synthesizing extracellular matrix compounds. It has been demonstrated that Pro-Hyp is generated at sites of skin wound healing.[22] The endogenous Pro-Hyp can enhance wound healing by promoting growth of p75NTR-positive fibroblasts. Ingestion of collagen hydrolysate has been shown to increase blood levels of Pro-Hyp.[13–17] The food-derived Pro-Hyp can also enhance wound healing by stimulating p75NTR-positive fibroblasts at sites of damage. However, the food-derived Pro-Hyp does not enhance growth of p75NTR-negative fibroblasts in the post-wound healing site or in normal tissues.

Therefore, ingestion of collagen hydrolysate enhances wound healing without inducing abnormal growth of resident fibroblasts.

Conclusions and Future Prospect

It has been demonstrated that ingestion of collagen hydrolysate improves skin and joint conditions[9,11,12] and enhances wound healing.[7,8] The ingestion of collagen hydrolysate increases collagen di- and tri- peptides such as Pro-Hyp, Hyp-Gly, and Pro-Hyp-Gly in human blood.[13–17] The present study demonstrates that most of meats in Japanese daily dish contain 0.5-2 g of collagen/gelatin. In some cases, more than 10 g collagen/gelatin can be taken by consumption of one serve of meat such as eel and shark tail meat. However, there is no report on content and structure of food-derived collagen peptides in blood after ingestion of meats. The present study demonstrates increase of collagen peptides in human blood after ingestion of collagen rich fish meat (shark tail meat). However, contents of collagen peptides in human blood after ingestion of collagen rich fish meat were approximately 30% of those after ingestion of collagen hydrolysate. Approximately 70% of the collagen in the shark meat remained insoluble after cooking and digestion with pepsin-pancreatin digestion. Therefore, only soluble peptide after endoproteinases digestion can be degraded by exopeptidases and absorbed in blood circulation system. To increase bioavailability of collagen peptide in meat, cooking method, which can increase solubility of collagen in meat, is necessary.

Previous studies have demonstrated that Pro-Hyp, a mainly food-derived collagen peptide in blood,[13–17] can trigger growth of fibroblast attached on collagen gel.[19] However, inconsistent results have been obtained when different preparation of FBS was used. The present study demonstrates presence of hydroxyprolyl peptides including Pro-Hyp in FBSs with different levels, which can mask effect of added Pro-Hyp. Therefore, FBS free from low molecular hydroxyprolyl peptide should be used to obtain reproducible data. The collagen peptides in FBS might play important role in development of fetus, as adult bovine serum contained hydroxyprolyl peptide in lower content than FBS. Approximately

30% of hydroxyprolyl peptides in FBS could not be identified. The unidentified peptides might be contained in human blood after ingestion of collagen hydrolysate and exert same biological function. These peptides should be identified and examined their function in future studies.

Some fibroblast cell lines did not respond to Pro-Hyp.[19] Excess growth of fibroblasts in body after ingestion of collagen hydrolysate has not been observed.[7–9] These facts suggest presence of Pro-Hyp responding and non-responding fibroblasts. To confirm this hypothesis, the mesenchymal stem cell marker p75NTR was used, as p75NTR-positive cells are increased at the wound healing site.[65] Most fibroblasts that had just migrated from mouse skin expressed p75NTR. After prolonged cultivation, however, the ratio of p75NTR-positive fibroblasts dropped. The p75NTR-positive fibroblasts specifically incorporated Pro-Hyp. These data clearly indicate presence of Pro-Hyp responding and non-responding fibroblasts. These results suggest that Pro-Hyp could enhance wound healing by stimulating only p75NTR-positive fibroblasts at wound healing sites, while it has little effect on p75NR-negative fibroblasts. Previous study has demonstrated that significant part of Pro-Hyp in tissue is modified in its proline residue without peptide bond hydrolysis.[68] As these peptides might affect growth signal of p75NTR-positive cell, these modified peptides should be identified in future studies. It is difficult to examine intracellular signaling by Pro-Hyp in p75NTR-positive fibroblast as fibroblast rapidly lose p75NTR and peptide transporter during cultivation. Further study on maintaining p75NTR is in progress.

The present study demonstrates that bioactive collagen peptides could be generated in blood not only after ingestion of collagen hydrolysate but also after ingestion of gelatin in cooked meat and the presence of Pro-Hyp responding and non-responding fibroblasts in the body, which could contribute a better understanding of wound healing process.

Acknowledgement

I would like to express the deepest appreciation to Dr. Kenji Sato, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, for his advice and guidance on a whole extent of research project in this thesis.

I would like to show my great gratitude to Dr. Haruhiko Toyohara and Dr. Masato Kinoshita, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, for their advices in our laboratory seminar giving insightful comments on this thesis.

I sincerely thank my current and former colleagues in Laboratory of Marine Biological Function, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, for their kind help and wide-ranging discussion.

I would like to express the appreciation to Dr. Sumi Sugiyama, Faculty of Human Culture and Science, Prefectural University of Hiroshima, for giving chance to go on the doctoral educational course.

Finally, I appreciate my family for supporting me throughout my Ph.D. years and my life in general.

References

(1) Ricard-Blum, S. The Collagen Family. *Cold Spring Harb. Perspect. Biol.* **2011**, *3* (1), 1–19.

(2) Alipour, H.; Raz, A.; Zakeri, S.; Dinparast Djadid, N. Therapeutic Applications of Collagenase (Metalloproteases): A Review. *Asian Pac. J. Trop. Biomed.* **2016**, *6* (11), 975–981.

(3) Gómez-Guillén, M. C.; Turnay, J.; Fernández-Díaz, M. D.; Ulmo, N.; Lizarbe, M. a.; Montero, P. Structural and Physical Properties of Gelatin Extracted from Different Marine Species: A Comparative Study. *Food Hydrocoll.* **2002**, *16* (1), 25–34.

(4) Sibilla, S.; Godfrey, M.; Brewer, S.; Budh-Raja, A.; Genovese, L. An Overview of the Beneficial Effects of Hydrolysed Collagen as a Nutraceutical on Skin Properties: Scientific Background and Clinical Studies. *Open Nutraceuticals J.* **2015**, *8* (1), 29–42.

(5) Gómez-Guillén, M. C.; Giménez, B.; López-Caballero, M. E.; Montero, M. P. Functional and Bioactive Properties of Collagen and Gelatin from Alternative Sources: A Review. *Food Hydrocoll.* **2011**, *25* (8), 1813–1827.

(6) Global Collagen Hydrolysate Market 2019: Product Overview and Scope, Growth Rate and Price Analysis by Type, Forecast to 2023 - MarketWatch https://www.marketwatch.com/press-release/global-collagen-hydrolysate-market-2019-product-overview-and-scope-growth-rate-and-price-analysis-by-type-forecast-to-2023-2019-06-04 (accessed Jan 21, 2020).

(7) Yamanaka, H.; Okada, S.; Sanada, H. A Multicenter, Randomized, Controlled Study of the Use of Nutritional Supplements Containing Collagen Peptides to Facilitate the Healing of Pressure Ulcers. *J. Nutr. Intermed. Metab.* **2017**, *8*, 51–59.

(8) Sugihara, F.; Inoue, N.; Venkateswarathirukumara, S. Ingestion of Bioactive Collagen Hydrolysates Enhanced Pressure Ulcer Healing in a Randomized Double-Blind Placebo-Controlled Clinical Study. *Sci. Rep.* **2018**, *8*, 11403.

(9) Kumar, S.; Sugihara, F.; Suzuki, K.; Inoue, N.; Venkateswarathirukumara, S. A Double-Blind, Placebo-Controlled, Randomised, Clinical Study on the Effectiveness of Collagen Peptide on Osteoarthritis. *J. Sci. Food Agric.* **2014**, *95* (4), 702–707.

(10) Benito-Ruiz, P.; Camacho-Zambrano, M. M.; Carrillo-Arcentales, J. N.; Mestanza-Peralta, M. A.; Vallejo-Flores, C. A.; Vargas-López, S. V.; Villacís-Tamayo, R. A.; Zurita-Gavilanes, L. A. A Randomized Controlled Trial on the Efficacy and Safety of a Food Ingredient, Collagen Hydrolysate, for Improving Joint Comfort. *Int. J. Food Sci. Nutr.* **2009**.

(11) Proksch, E.; Segger, D.; Degwert, J.; Schunck, M.; Zague, V.; Oesser, S. Oral Supplementation of Specific Collagen Peptides Has Beneficial Effects on Human Skin Physiology: A Double-Blind, Placebo-Controlled Study. *Skin Pharmacol. Physiol.* **2014**, *27* (1), 47–55.

(12) Proksch, E.; Schunck, M.; Zague, V.; Segger, D.; Degwert, J.; Oesser, S. Oral Intake of Specific Bioactive Collagen Peptides Reduces Skin Wrinkles and Increases Dermal Matrix Synthesis. *Skin Pharmacol. Physiol.* **2014**, *27* (3), 113–119.

(13) Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K. Identification of Food-Derived Collagen Peptides in Human Blood after Oral Ingestion of Gelatin Hydrolysates. *J. Agric. Food Chem.* **2005**, *53* (16), 6531–6536.

(14) Shigemura, Y.; Suzuki, A.; Kurokawa, M.; Sato, Y.; Sato, K. Changes in Composition and Content of Food-Derived Peptide in Human Blood after Daily

Ingestion of Collagen Hydrolysate for 4 Weeks. *J. Sci. Food Agric.* **2018**, *98* (5), 1944–1950.

(15) Ohara, H.; Matsumoto, H.; Ito, K.; Iwai, K.; Sato, K. Comparison of Quantity and Structures of Hydroxyproline-Containing Peptides in Human Blood after Oral Ingestion of Gelatin Hydrolysates from Different Sources. *J. Agric. Food Chem.* **2007**, *55* (4), 1532–1535.

(16) Ichikawa, S.; Morifuji, M.; Ohara, H.; Matsumoto, H.; Takeuchi, Y.; Sato, K. Hydroxyproline-Containing Dipeptides and Tripeptides Quantified at High Concentration in Human Blood after Oral Administration of Gelatin Hydrolysate. *Int. J. Food Sci. Nutr.* **2010**, *61* (1), 52–60.

(17) Shigemura, Y.; Kubomura, D.; Sato, Y.; Sato, K. Dose-Dependent Changes in the Levels of Free and Peptide Forms of Hydroxyproline in Human Plasma after Collagen Hydrolysate Ingestion. *Food Chem.* **2014**, *159*, 328–332.

(18) Shigemura, Y.; Akaba, S.; Kawashima, E.; Park, E. Y.; Nakamura, Y.; Sato, K. Identification of a Novel Food-Derived Collagen Peptide, Hydroxyprolyl-Glycine, in Human Peripheral Blood by Pre-Column Derivatisation with Phenyl Isothiocyanate. *Food Chem.* **2011**, *129* (3), 1019–1024.

(19) Shigemura, Y.; Iwai, K.; Morimatsu, F.; Iwamoto, T.; Mori, T.; Oda, C.; Taira, T.; Park, E. Y.; Nakamura, Y.; Sato, K. Effect of Prolyl-Hydroxyproline (Pro-Hyp), a Food-Derived Collagen Peptide in Human Blood, on Growth of Fibroblasts from Mouse Skin. *J. Agric. Food Chem.* **2009**, *57* (2), 444–449.

(20) Kimira, Y.; Ogura, K.; Taniuchi, Y.; Kataoka, A.; Inoue, N.; Sugihara, F.; Nakatani, S.; Shimizu, J.; Wada, M.; Mano, H. Collagen-Derived Dipeptide Prolyl-Hydroxyproline Promotes Differentiation of MC3T3-E1 Osteoblastic Cells. *Biochem. Biophys. Res. Commun.* **2014**, *453* (3), 498–501.

(21) Kusubata, M.; Koyama, Y. I.; Tometsuka, C.; Shigemura, Y.; Sato, K. Detection of Endogenous and Food-Derived Collagen Dipeptide Prolylhydroxyproline

(Pro-Hyp) in Allergic Contact Dermatitis-Affected Mouse Ear. *Biosci. Biotechnol. Biochem.* **2015**, *79* (8), 1356–1361.

(22) Jimi, S.; Sato, K.; Kimura, M.; Suzumiya, J.; Hara, S.; De Francesco, F.; Ohjimi, H. G-CSF Administration Accelerates Cutaneous Wound Healing Accompanied With Increased Pro-Hyp Production In Db/Db Mice. *Clin. Reseach Dermatol.* **2017**.

(23) Mizushige, T.; Nogimura, D.; Nagai, A.; Mitsuhashi, H.; Taga, Y.; Kusubata, M.; Hattori, S.; Kabuyama, Y. Ginger-Degraded Collagen Hydrolysate Exhibits Antidepressant Activity in Mice. *J. Nutr. Sci. Vitaminol. (Tokyo).* **2019**, *65* (3), 251–257.

(24) Yamamoto, S.; Hayasaka, F.; Deguchi, K.; Okudera, T.; Furusawa, T.; Sakai, Y. Absorption and Plasma Kinetics of Collagen Tripeptide after Peroral or Intraperitoneal Administration in Rats. *Biosci. Biotechnol. Biochem.* **2015**, *79* (12), 2026–2033.

(25) Yazaki, M.; Ito, Y.; Yamada, M.; Goulas, S.; Teramoto, S.; Nakaya, M.; Ohno, S.; Yamaguchi, K. Oral Ingestion of Collagen Hydrolysate Leads to the Transportation of Highly Concentrated Gly-Pro-Hyp and Its Hydrolyzed Form of Pro-Hyp into the Bloodstream and Skin. *J. Agric. Food Chem.* **2017**, *65* (11), 2315–2322.

(26) Yamamoto, S.; Deguchi, K.; Onuma, M.; Numata, N.; Sakai, Y. Absorption and Urinary Excretion of Peptides after Collagen Tripeptide Ingestion in Humans. *Biol. Pharm. Bull.* **2016**, *39* (3), 428–434.

(27) Ohara, H.; Iida, H.; Ito, K.; Takeuchi, Y.; Nomura, Y. Effects of Pro-Hyp, a Collagen Hydrolysate-Derived Peptide, on Hyaluronic Acid Synthesis Using in Vitro Cultured Synovium Cells and Oral Ingestion of Collagen Hydrolysates in a Guinea Pig Model of Osteoarthritis. *Biosci. Biotechnol. Biochem* **2010**, *74* (10), 2096–2099.

(28) Nakatani, S.; Mano, H.; Sampei, C.; Shimizu, J.; Wada, M. Chondroprotective Effect of the Bioactive Peptide Prolyl-Hydroxyproline in Mouse Articular Cartilage in Vitro and in Vivo. *Osteoarthr. Cartilage* **2009**, *17* (12), 1620–1627.

(29) Sato, K.; Yoshinaka, R.; Sato, M.; Shimizu, Y. Collagen Content in the Muscle of Fishes in Association with Their Swimming Movement and Meat Texture. *Nippon Suisan Gakkaishi* **1986**, *52* (9), 1595–1600.

(30) Casey, J. C.; Crosland, A. R.; Patterson, R. L. S. Collagen Content of Meat Carcasses of Known History. *Meat Sci.* **1986**, *12* (4), 189–203.

(31) Liu, A.; Nishimura, T.; Takahashi, K. *Relationship Between Structural Properties of Intramuscular Connective Tissue and Toughness of Various Chicken Skeletal Muscles*; ELSEVIER, 1996; Vol. 43.

(32) Ministry of Health Labour and Welfare (Japan). Japan the National Health and Nutrition Survey in Japan 2012 https://www.mhlw.go.jp/bunya/kenkou/eiyou/dl/h25-houkoku-04.pdf (accessed Aug 25, 2018).

(33) Une, S. Dietary Customs Related to Shark Cuisine from the Meiji Era to the Present. *J. Cook. Sci. Jpn.* **2015**, *48* (4), 308–319.

(34) Kumakura, I.; Fushiki, T. *Introduction to Japanese Cuisine : Nature, History and Culture*; LETRAS, Ed.; Shuhari Initiative Ltd., 2015.

(35) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid Analysis of Amino Acids Using Pre-Column Derivatization. *J. Chromatogr.* **1984**, *336* (1), 93–104.

(36) Lin, Y. K.; Liu, D. C. Comparison of Physical–Chemical Properties of Type I Collagen from Different Species. *Food Chem.* **2006**, *99* (2), 244–251.

(37) Yata, M.; Yoshida, C.; Fujisawa, S.; Yoshinaka, R. Identification and Characterization of Molecular Species of Collagen in Fish Skin. *Food Chem. Toxicol.* **2001**, *66* (2), 247–251.

(38) Nagai, T.; Izumi, M.; Ishii, M. Fish Scale Collagen. Preparation and Partial Characterization. *Int. J. Food Sci. Technol.* **2004**, *39* (3), 239–244.

(39) Kimura, S.; Zhu, X.; Matsui, R.; Shijoh, M.; Satoshi, T. Characterization of Fish Muscle Type I Collagen. *J. Food Sci.* **1988**, *53* (5), 1315–1318.

(40) Mizuta, S.; Yoshinaka, R.; Sato, M.; Sakaguchi, M. Isolation and Partial Characterization of Two Distinct Types of Collagen in the Squid Todarodes Pacificus. *Fish. Sci.* **1994**, *60* (4), 467–471.

(41) Limpisophon, K.; Tanaka, M.; Weng, W.; Abe, S.; Osako, K. Characterization of Gelatin Films Prepared from Under-Utilized Blue Shark (Prionace Glauca) Skin. *Food Hydrocoll.* **2009**, *23* (7), 1993–2000.

(42) Ohara, H.; Ichikawa, S.; Matsumoto, H.; Akiyama, M.; Fujimoto, N.; Kobayashi, T.; Tajima, S. Collagen-Derived Dipeptide, Proline-Hydroxyproline, Stimulates Cell Proliferation and Hyaluronic Acid Synthesis in Cultured Human Dermal Fibroblasts. *J. Dermatol.* **2010**, *37* (4), 330–338.

(43) Noguchi, C.; Kobayashi, M.; Koyama, Y. Amount of Collagen Ingested by Japanese Adult Women from Their Diet. *Japanese J. Nutr. Diet.* **2012**, *70* (2), 120–128.

(44) Furuichi, T.; Komekura, M.; Suzuki, M.; Asai, T.; Sugiyama, S. Collagen Characteristics and Textural Properties of Chicken Wing Sticks Prepared in a Cook-Chill System Using a Cooking Pan or Vacuum Package. *J. Cook. Sci. Jpn.* **2013**, *46* (6), 372–380.

(45) Lin, Y. K.; Liu, D. C. Comparison of Physical-Chemical Properties of Type I Collagen from Different Species. *Food Chem.* **2006**, *99* (2), 244–251.

(46) Fu, Y.; Young, J. F.; Therkildsen, M. Bioactive Peptides in Beef : Endogenous Generation through Postmortem Aging. *Meat Sci*. **2017**, *123*, 134–142.

(47) Sato, K.; Ando, M.; Kubota, S.; Origasa, K.; Kawase, H.; Toyohara, H.; Sakaguchi, M.; Nakagawa, T.; Makinodan, Y.; Ohtsuki, K.; Kawabata M.

Involvement of Type V Collagen in Softening of Fish Muscle during Short-Term Chilled Storage. *J. Agric. Food Chem.* **1997**, *45* (2), 343–348.

(48) Lee, S. K.; Posthauer, M. E.; Dorner, B.; Redovian, V.; Maloney, M. J. Pressure Ulcer Healing with a Concentrated, Fortified, Collagen Protein Hydrolysate Supplement: A Randomized Controlled Trial. *Adv. Skin Wound Care* **2006**, *19* (2), 92–96.

(49) Kono, T.; Tanii, T.; Furukawa, M.; Mizuno, N.; Kitajima, J.; Ishii, M.; Hamada, T.; Yoshizato, K. Cell Cycle Analysis of Human Dermal Fibroblasts Cultured on or in Hydrated Type I Collagen Lattices. *Arch. Dermatol. Res.* **1990**, *282* (4), 258–262.

(50) Price, P. J.; Gregory, E. A. Relationship between in Vitro Growth Promotion and Biophysical and Biochemical Properties of the Serum Supplement. *In Vitro* **1982**, *18* (6), 576–584.

(51) Fabricant, R. N.; De Larco, J. E.; Todaro, G. J. Nerve Growth Factor Receptors on Human Melanoma Cells in Culture. *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74* (2), 565–569.

(52) Tomellini, E.; Lagadec, C.; Polakowska, R.; Le Bourhis, X. Role of P75 Neurotrophin Receptor in Stem Cell Biology: More than Just a Marker. *Cell. Mol. Life Sci.* **2014**, *71* (13), 2467–2481.

(53) Álvarez-Viejo, M. CD271 as a Marker to Identify Mesenchymal Stem Cells from Diverse Sources before Culture. *World J. Stem Cells* **2015**, *7* (2), 470–476.

(54) Pincelli, C. P75 Neurotrophin Receptor in the Skin: Beyond Its Neurotrophic Function. *Front. Med.* **2017**, *4* (MAR), 1–8.

(55) Trim, N.; Morgan, S.; Evans, M.; Issa, R.; Fine, D.; Afford, S.; Wilkins, B.; Iredale, J. Hepatic Stellate Cells Express the Low Affinity Nerve Growth Factor Receptor P75 and Undergo Apoptosis in Response to Nerve Growth Factor Stimulation. *Am. J. Pathol.* **2000**, *156* (4), 1235–1243.

(56) Nakamura, T.; Endo, K.; Kinoshita, S. Identification of Human Oral Keratinocyte Stem/Progenitor Cells by Neurotrophin Receptor P75 and the Role of Neurotrophin/P75 Signaling. *Stem Cells* **2006**, *25* (3), 628–638.

(57) Palazzo, E.; Marconi, A.; Truzzi, F.; Dallaglio, K.; Petrachi, T.; Humbert, P.; Schnebert, S.; Perrier, E.; Dumas, M.; Pincelli, C. Role of Neurotrophins on Dermal Fibroblast Survival and Differentiation. *J. Cell. Physiol.* **2012**, *227* (3), 1017–1025.

(58) Meeker, R.; Williams, K. Dynamic Nature of the P75 Neurotrophin Receptor in Response to Injury and Disease. *J. Neuroimmune Pharmacol.* **2014**, *9* (5), 615–628.

(59) Kimberly S. Williams, Deirdre A. Killebrew, Gillian P. Clary, Jaimie A. Seawell, and R. B. M. Differential Regulation of Macrophage Phenotype by Mature and Pro-Nerve Growth Factor. *J Neuroimmunol.* **2015**, *285*, 76–93.

(60) Yamada, T.; Akamatsu, H.; Hasegawa, S.; Yamamoto, N.; Yoshimura, T.; Hasebe, Y.; Inoue, Y.; Mizutani, H.; Uzawa, T.; Matsunaga, K.; et al. Age-Related Changes of P75 Neurotrophin Receptor-Positive Adipose-Derived Stem Cells. *J. Dermatol. Sci.* **2010**, *58* (1), 36–42.

(61) Hasebe, Y.; Hasegawa, S.; Hashimoto, N.; Toyoda, M.; Matsumoto, K.; Umezawa, A.; Yagami, A.; Matsunaga, K.; Mizutani, H.; Nakata, S.; et al. Analysis of Cell Characterization Using Cell Surface Markers in the Dermis. *J. Dermatol. Sci.* **2011**, *62* (2), 98–106.

(62) Abe, H.; Satoh, M.; Miyauchi, S.; Shuto, S.; Matsuda, A.; Kamo, N. Conjugation of Dipeptide to Fluorescent Dyes Enhances Its Affinity for a Dipeptide Transporter (PEPT1) in Human Intestinal Caco-2 Cells. *Bioconjug. Chem.* **1999**, *10* (1), 24–31.

(63) Kudo, M.; Katayoshi, T.; Kobayashi-Nakamura, K.; Akagawa, M.; Tsuji-Naito, K. H+/Peptide Transporter (PEPT2) Is Expressed in Human Epidermal

Keratinocytes and Is Involved in Skin Oligopeptide Transport. *Biochem. Biophys. Res. Commun.* **2016**, *475* (4), 335–341.

(64) Sabine, W.; Richard, G. Regulation of Wound Healing by Growth Factors and Cytokines. *Physiol. Rev* **2003**, *83*, 835–870.

(65) Iwata, Y.; Akamatsu, H.; Hasegawa, S.; Takahashi, M. The Epidermal Integrin Beta-1 and P75NTR Positive Cells Proliferating and Migrating during Wound Healing Produce Various Growth Factors , While the Expression of P75NTR Is Decreased in Patients with Chronic Skin Ulcers. *J. Dermatol. Sci.* **2013**, *71* (2), 122–129.

(66) Micera, A.; Vigneti, E.; Pickholtz, D.; Reich, R.; Pappo, O.; Bonini, S.; Maquart, F. X.; Aloe, L.; Levi-Schaffer, F. Nerve Growth Factor Displays Stimulatory Effects on Human Skin and Lung Fibroblasts, Demonstrating a Direct Role for This Factor in Tissue Repair. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (11), 6162–6167.

(67) Iwata, Y.; Hasebe, Y.; Hasegawa, S.; Nakata, S.; Yagami, A.; Matsunaga, K.; Sugiura, K.; Akamatsu, H. Dermal CD271+ Cells Are Closely Associated with Regeneration of the Dermis in the Wound Healing Process. *Acta Derm. Venereol.* **2017**, *97* (5), 593–600.

(68) Kawaguchi, T.; Nanbu, P. N.; Kurokawa, M. Distribution of Prolylhydroxyproline and Its Metabolites after Oral Administration in Rats. *Biol. Pharm. Bull.* **2012**, *35* (3), 422–427.