The Protective Effects of Long-Term Oral Administration of Marine Collagen Hydrolysate from Chum Salmon on Collagen Matrix Homeostasis in the Chronological Aged Skin of Sprague-Dawley Male Rats

Jiang Liang, Xinrong Pei, Zhaofeng Zhang, Nan Wang, Junbo Wang, and Yong Li

Abstract: To investigate the long-term effects of marine collagen hydrolysate (MCH) from Chum Salmon skin on the aberrant collagen matrix homeostasis in chronological aged skin, Sprague-Dawley male rats of 4-wk-old were orally administered with MCH at the diet concentrations of 2.25% and 4.5% for 24 mo. Histological and biochemical analysis revealed that MCH had the potential to inhibit the collagen loss and collagen fragmentation in chronological aged skin. Based on immunohistochemistry and western blot analysis, collagen type I and III protein expression levels in MCH-treated groups significantly increased as compared with the aged control group. Furthermore, quantitative real-time polymerase chain reaction and western blot analysis showed MCH was able to increase the expressions of procollagen type I and III mRNA (COL1A2 and COL3A1) through activating Smad signaling pathway with up-regulated TGF-βRII (TβRII) expression level. Meanwhile, MCH was shown to inhibit the age-related increased collagen degradation through attenuating MMP-1 expression and increasing tissue inhibitor of metalloproteinases-1 expression in a dose-dependent manner. Moreover, MCH could alleviate the oxidative stress in chronological aged skin, which was revealed from the data of superoxide dismutase activity and the thiobarbituric acid reactive substances level in skin homogenates. Therefore, MCH was demonstrated to have the protective effects on chronological skin aging due to the influence on collagen matrix homeostasis. And the antioxidative property of MCH might play an important role in the process.

Keywords: chronological aging, collagen hydrolysate, collagen matrix, skin, Sprague-Dawley rat

Introduction

Compared with the ultraviolet-induced or extrinsic skin aging, chronological or intrinsic skin aging is a cumulative process depending on the passage of time (Fisher and others 2002). Histologically, chronological aged skin is characterized by the decreased thickness of dermal matrix (El-Domyati and others 2002). The principal structural components of extracellular matrix in dermis consist of fibril-forming collagens. The predominant form of collagen in dermis is type I (85% to approximately 90%), followed by small amounts of type III (10% to approximately 15%) (Chung and others 2001). Type I collagen is characterized by thick fiber that confer stiffness and resistance to perform a crucial function in maintaining the structure of dermis. Whereas collagen type III is characterized by thin fiber that present the resiliency of skin. Collagen fibers arrange parallel to skin surface and are responsible for the high tensile strength and resiliency of skin (Tayebjee and others 2003).

In sun-protected young skin, the synthesis and degradation of collagen are balanced to maintain the collagen content and structural integrity of skin. The collagen biosynthesis can be primarily regulated by a family of transforming growth factor-β (TGF-β) through the Smad signaling pathway. The Smads are a series of proteins that perform downstream functions from the serine/threonine kinase receptors of the TGF-β family, thereby propagating signals to the nucleus. The combination of TGF-β to the TGF-β receptor type II (TβRII) is the initial step to activate the intrinsic serine/threonine kinase activity of the TGF-β receptor type I that triggers the phosphorylation and activation of Smad2 and Smad3. Meanwhile, the activation of Smad2 and Smad3 are antagonized by the endogenous negative regulators Smad6 and Smad7 (Moustakas and others 2001). The degradation of type I and III fibrillar collagens is initiated by matrix metalloproteinases-1 (MMP-1), which belongs to the metalloproteinases (MMPs), a large family of zinc-dependent endo-proteases with a broad range of substrate specificities and the capacity of degrading all extracellular matrix proteins. On the other hand, the activity of MMP-1 is inhibited by the specific endogenous tissue inhibitor of metalloproteinases-1 (TIMP-1) (Vise and Nagase 2003). In the chronological aged skin, collagen homeostasis is aberrant with molecular features of the decreased synthesis of type I and III procollagens (the precursors to collagens) and elevated degradation of mature collagen fibers, which result in general atrophy of the...
extracellular matrix and impaired collagen fibril organization (Rittie and Fisher 2002).

In addition, sustained age-associated oxidative damages to DNA, proteins, and membrane lipids lead to the accumulation of thiobarbituric acid reactive substances (TBARs), the products of lipid peroxidation and other breakdown products from oxidatively modified proteins, carbohydrates, and nucleic acids (Lovell and others 1997; Fisher and others 2001). Meanwhile, the antioxidant defense is also attenuated in the chronological aged skin (Rittie and Fisher 2002). Superoxide dismutase (SOD), which can effectively scavenge reactive oxygen species (ROS) and inhibit lipid peroxidation, plays an important role in the maintenance of cellular redox homeostasis and protects skin cells from oxidative damage (Shindo and others 1993; Murakami and others 2009). Increased oxidative stress can lead to the dysregulated intracellular and extracellular homeostasis that can modify cellular behavior and cell matrix interactions. Moreover, ROS can directly induce MMPs transcription and activate these enzymes (Nelson and Melendez 2004; Fisher and others 2009). Therefore, the age-related increased oxidative stress is confirmed to play an important role in regulating collagen matrix metabolism during skin aging (Yasui and Sakurai 2000; Siwik and others 2001).

As we know, collagen or gelatin (the partially hydrolyzed collagen) in dietary supplementation has been verified to involve in the extracellular matrix synthesis for improving joint, nail, and hair conditions (Moskowitz 2000; Zague 2008). As the further polypeptide composite enzymatically hydrolyzed from gelatin, collagen hydrolysate is characterized by relative lower molecular weight distribution (<6 kDa), higher absorption and bioavailability, as well as a wider range of functional and biological properties, including antioxidant and antihypertensive activities (Kim and others 2001; Morimura and others 2002). The collagen hydrolysate can be traditionally isolated from the skin of land-based animals, such as cow and pig (Wang and others 2008). With marine species comprising nearly one-half of the total global biodiversity, the skin, scale, and bone of the marine life, especially the various fish species have become new sources of collagen hydrolysate (Wang and others 2008). In recent years, there is a growing interest in the potentially beneficial effects of marine collagen hydrolysate (MCH) on skin aging. Although series of studies have illustrated the antioxidative property of collagen hydrolysate from marine fish contributed to the protective effects on UV-induced skin photo-aging in vivo (Hou and others 2009; Zhuang and others 2009), the effects of marine fish collagen hydrolysate on intrinsic or chronological skin aging as well as the related molecular mechanisms remain to be elucidated. Moreover, as we know, fish collagen hydrolysates from different species, habitats and tissues are significantly different in terms of properties because of the unique amino acid composition and the variation in the amount of amino acids (Wang and others 2008). At the present time, there has been no report on the antichronological skin aging effects of MCH from Chum Salmon, the deep sea fish widely distributed in the north Pacific and Atlantic. Our previous study has revealed the protective effect of 90-d oral ingestion of MCH from Chum Salmon on D-galactose induced rapid intrinsic skin aging in mice (Pei and others 2008). Further research still needs to be conducted to confirm the long-term effect of MCH on chronological skin aging. Therefore, in this study, we initially investigated the effects of long-term (24 mo) oral ingestion of MCH from Chum Salmon (Oncorhynchus keta) on the collagen content and collagen homeostasis in the chronological aged skin of male Sprague-Dawley (S-D) rat. These experiments could help us to make more effective use of collagen hydrolysate from Chum Salmon (O. keta) skin and explore its potentials in commercial applications, as antiaging functional ingredients in foods, cosmetics, and biomedical materials.

Materials and Methods

Preparation and identification procedure of test substance

MCH provided by CF Haishi Biotechnology Ltd. Co. (Beijing, China) was prepared from the skin of wild-caught Chum Salmon (O. keta) (from the East China Sea, average body weight, 1.47 kg). The procedures for MCH preparation and identification were according to the method described in our previous research (Pei and others 2010). Briefly, the fish skin was cleaned, scaled, cut into small pieces, defatted, and homogenized. After being homogenized and emulsified in distilled water, the material was hydrolyzed by complex protease (3000 U/g protein) including 7% of trypsin, 65% of papain, and 28% of alkaline proteinase, at 40 °C and pH 8 for 3 h. The resultant hydrolysate was reextracted by centrifugation and subsequently separated through ceramic membrane (200 μm). After being purified through a procedure of nanofiltration and condensed by cryoconcentration under vacuum at 70 °C with an evaporation rate of 500 kg/h, decolorized with active carbon at 75 °C for 1 h, filtrated and dried by spray drying with the pressure of 20 MPa at an evaporation rate of 200 kg/h, MCH powder used in the following investigations was obtained. Then the molecular weight distribution of the sample was analyzed by the coupling technique of high-performance liquid chromatography (HPLC, Waters Corp., Milford, Mass., U.S.A.) and LDI-1700 matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Linier Scientific Inc., Reno, Nev., U.S.A.). The amino acid composition was analyzed with an amino acid analyzer (H835–50; Hitachi, Tokyo, Japan).

Animals

Young weaning male S-D rats, 4-wk-old, weighing 80 to approximately 90 g, were acquired from the Animal Service of Health Science Center, Peking Univ.. Two rats per plastic cage were housed with free access to chow and tap water and maintained in a filter-protected air-conditioned room with controlled temperature (25 ± 2 °C), relative air humidity (60 ± 5%) and 12 h light/dark cycles (light on 07:30–19:30 h). After the acclimatization period of 1 wk, the animals were randomly distributed into 3 groups, that is, the vehicle control group (n = 20), fed with the AIN93M rodent chow from Vital River Ltd., Co. (Beijing, China) comprising 25% of crude protein, 4.24% of crude fat, and 55.19% of carbohydrate. The animals of 2 experimental groups (n = 20 per group) were treated with MCH added in the AIN93M diet, in which the equivalent proportion of crude protein was replaced by MCH at the concentration of 2.25% and 4.5% (wt/wt), respectively. Body weight and food consumption were recorded weekly in the first 6 mo and every 2 wk thereafter until euthanasia. No mortality was found in any groups treated with MCH for 12 mo. At the age of 25 mo, the survival rates of control, 2.25% and 4.5% MCH groups were 62%, 70%, and 72%, respectively. Ten rats from each group were anesthetized with an intraperitoneal injection of pentobarbital sodium (45 mg/kg body weight) and sacrificed. Moreover, during the experiment, another group of male S-D rats (n = 10) fed with the control diet was sacrificed at the age of 12 mo to be used as the middle-aged controls.
After sacrifice, full thickness skin samples about 4 cm$^2$ were immediately excised from the left depilated dorsal regions, trimmed off loose connective tissue and musculature, snap-frozen in liquid nitrogen, and then stored at $-80 \degree$C until analysis. Full thickness skin samples about 4 cm$^2$ excised from the right depilated dorsal regions were fixed in 10% buffered formalin for histological examinations and measurements.

All animals were handled in accordance with the guidelines of the Principle of Laboratory Animal Care (NIH Publication No. 85–23, revised 1985) and the guidelines of the Peking Univ. Animal Research Committee.

Histological measurements

Skin samples fixed in 10% buffered formalin for at least 24 h were processed for processing for conventional paraffin embedding. Then the de-paraffinized sections of 5 μm thickness were processed for Mason’s trichrome stain to localize collagen. Dermal thickness was determined by measuring the distance between the epidermal–dermal junction and the dermal–fat junction at 5 randomly selected fields (40× microscopic magnifications) from 3 slides per sample in each animal using Image pro-plus 6.0 (Media Cybernetics, Inc., Bethesda, Md., U.S.A.). To prevent observer bias, all specimens were coded and examined without knowledge of experimental conditions.

Assessment of total collagen by hydroxyproline method

The total collagen content was calculated using the assumption that hydroxyproline accounts for 13% of collagen (Babraj and others 2005). Skin samples stored at $-80 \degree$C were defatted and dried to a constant weight. Determination of hydroxyproline content in the dried skin sample from dorsal region was carried out according to the protocol of a commercial hydroxyproline detection kit (Nanjing Jiancheng Biotechnology Co., Nanjing, China). Briefly, the samples were hydrolyzed by sodium hydroxide for 20 min at 100 °C and oxidized by chloramine-T. The oxidized products could react with dimethyl-amino-benzaldehyde to form the end-product with the maximal absorption at 550 nm. The amount of hydroxyproline in each sample was calculated by comparison with the absorbance of the hydroxyproline standard and was expressed as microgram hydroxyproline per milligram dry tissue sample.

Immunohistochemistry

Immunohistochemistry was performed on 10% buffered formalin solution fixed, paraffin embedded tissue sections using a Real Envision Detection ABC kit (Code nr Gk500705; GenTech [Shanghai] Company Ltd, Shanghai, China). The detailed procedure was performed according to the manufacturers’ instructions. In brief, the deparaffinized and transferred 5 μm thickness slides were incubated in a mixture of methanol and 3% hydrogen peroxide to block the activity of endogenous peroxidase. For antigen retrieval, the slides were boiled in 0.01 mmol/L sodium citrate (pH 6.0) buffer solutions for 15 min. To reduce nonspecific background staining, slides were incubated in goat serum albumin at room temperature for 30 min. Then the sections were incubated with the primary antibodies (mouse monoclonal anti-Collagen I at 1:700 [Abcam, Cambridge, UK] and mouse monoclonal anti-Collagen III at 1:500 [Abcam]) at 4 °C overnight. The sections were then incubated at room temperature with goat-anti-mouse EnVision–HRP (Horseradish Peroxidase)–enzyme for 120 min.

The immunoreactivity was visualized with 3,3′-diaminobenzidine chromogens. The sections were counterstained with hematoxylin and viewed under an Olympus BH-2 microscope (Olympus Corp., Tokyo, Japan) with images obtained by digital capture.

Real-time quantitative PCR analysis

The isolation of total RNA from the skin sample (about 50 mg) was according to the manufacturers’ protocol of the RNasey® fibrous tissue mini kit (Qiagen). Total RNA was quantified by 30 to 50 μL RNase-free dH$_2$O and analyzed for quantity and quality using a spectrophotometer.

Total RNA was reverse transcribed in a 25 μL reaction using the M-MLV Reverse Transciptase (Promega M170A). Two microliter of the resulting cDNA was then used in the real-time PCR step that was performed using BioEasy SYBR Green I Real Time PCR Kit Manual in the Line–gene fluorescence quantitative PCR detection system (Hangzhou Biero Technology Co., Ltd., Hangzhou, China). The primers used were as follows: Rattus β-actin (NM-031144.2, 150 bp), 5′-CCCCATCTATGAGGGTTACGC-3′ and 5′-TTTAATGTC ACGCAAGATTTC-3′; Rattus COL1A2 (NM-053356, 118 bp), 5′-GCCGTTGCAATATGATCCA-3′ and 5′-TGCAATGTTACACACG-3′; Rattus COL3A1 (X70369, 81 bp), 5′-CCATTGGCTGAGTGAGGT-3′ and 5′-TGTTGATCTTTGAATCATCATTGAGAT-3′. The relative change in mRNA expression level was determined by the 2$^ΔΔCt$ method with β-actin as the reference gene.

Western blot analysis

Frozen skin sample (about 100 mg) was cut into small pieces (about 1 mm$^3$) and powdered in a liquid nitrogen bath. Added with about 1 mL tissue lysis solution (Try [50 mM], Triton X-100 [0.1%], NaCl [150 mM], and EGTA [ethylene glycol tetraacetic acid]/EDTA [ethylene diaminetetraacetic acid; 2 mM], pH 7.4, containing mammalian protease inhibitor cocktail (1:100 dilution), sodium pyrophosphate [1 mM], phenylmethylsulfonyl fluoride [PMSF; 10 μg/mL], sodium vanadate [1 mM], and sodium fluoride [50 mM]), the sample was homogenized with tissue homogenizer (XHF-1, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for three 5 s pulses at 5000 rpm in an ice bath. Homogenates were then centrifuged at 12500 rpm for 5 min at 4 °C to collect the supernatants. Total protein concentration was determined by the BCA assay kit (Pierce Biotechnology, Ill., U.S.A.). Equal amounts (40 μg) of total protein per sample and prestained molecular weight standards were separated by Tris-glycine gels (Collagen I, 8% gel; Collagen III, 10% gel; TGF/β II, 10% gel; Smad2, 10% gel; Smad3, 10% gel; Smad7, 12% gel; TIMP-1, 15% gel; MMP-1, 10% gel; β-actin, 10% gel) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Mass., U.S.A.).

The membranes were blocked by 5% skin milk powder in Tris-buffered saline with 0.1% Tween–20 for 2 h at room temperature and incubated overnight at 4 °C with the following antibodies: mouse monoclonal anti-Collagen I (1:1500; Abcam), mouse monoclonal anti-Collagen III (1:500; Abcam), rabbit polyclonal anti-TGF/βRII (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., U.S.A.), goat polyclonal anti-Smad2 (1:200; Santa Cruz), rabbit polyclonal antiphospho-Smad3 (1:200; Santa Cruz), mouse monoclonal anti-Smad3 (1:200; Santa Cruz), rabbit polyclonal antiphospho-Smad3 (1:1000; Millipore); goat polyclonal anti-Smad7 (1:200; Santa Cruz), rabbit polyclonal anti-MMP-1 (1:100; Boster, Wuhan, China), rabbit polyclonal anti-TIMP-1 (1:200; Santa Cruz, CA, USA), and rabbit polyclonal anti-β-actin.
(1:200; Santa Cruz, CA, USA). After being washed in Tween-TBS (the self-made reagent of Tris Buffered Saline with 0.05% Tween® 20), membranes were incubated for 1 h at 37 °C with the appropriate HRP-conjugated secondary antibodies. The target proteins were visualized using enhanced chemiluminescence reagents (ECL kit; Millipore) and developed using Kodak films. The intensity of each band was quantified using public domain National Institutes of Health (NIH) Image Program (http://rsb.info.nih.gov/nih-image/download.html) and normalized using β-actin as a marker for equal protein loading.

Measurement of oxidative status in skin

About 100 mg skin sample was homogenized with 9 volumes (w/v) of phosphate buffered saline (PBS, pH 7.0) using tissue homogenizer (XHF-1, Ningbo Scientz Biotechnology Co., Ltd.) for three 5 s pulses at 5000 rpm at 4 °C. The SOD activity and TBARS level in the supernatant of tissue homogenates were determined according to the manufacturers’ instructions of the commercial kits purchased from Jiancheng Inst. of Biotechnology (Nanjing, China).

Statistical analyses

Statistical analyses were performed using SPSS (Version 13.0, SPSS Inc., Ill., U.S.A.). All variances in the measurement data expressed as mean ± standard error of mean (SEM) were checked for homogeneity by the Bartlett’s test. The one-way ANOVA test was used to analyze the group difference by least significant difference (LSD) (equal variance assumed) or Tamhane’s T2 (equal variances not assumed) post hoc tests. A value of P < 0.05 was considered significant.

Results

Characterization of MCH

The analysis of MCH indicated 86.5% of peptides with the molecular weights distributed between 130 and 1000 Da, and 91.5% below 1000 Da. The result of amino acid composition in Table 1 shows that MCH was rich in Gly > Glu > Pro > Hyp > Asp > Ala > Arg. Furthermore, MCH was revealed to contain very little or no carbohydrate by negative staining the polycrylamide gel with periodic acid-Schiff reagent (data not shown).

Table 1–Amino acid composition of MCH from Chum Salmon skin.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Nr residues/100 residues</th>
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<tbody>
<tr>
<td>Glycine</td>
<td>23.77</td>
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<tr>
<td>Glutamic acid</td>
<td>12.32</td>
</tr>
<tr>
<td>Proline</td>
<td>9.79</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>7.51</td>
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<tr>
<td>Aspartic acid</td>
<td>7.29</td>
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<tr>
<td>Alanine</td>
<td>6.59</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.08</td>
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<tr>
<td>Lysine</td>
<td>5.66</td>
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<tr>
<td>Leucine</td>
<td>4.64</td>
</tr>
<tr>
<td>Serine</td>
<td>4.23</td>
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<tr>
<td>Valine</td>
<td>2.94</td>
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<tr>
<td>Isoleucine</td>
<td>2.57</td>
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<tr>
<td>Threonine</td>
<td>2.53</td>
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<tr>
<td>Phenylalanine</td>
<td>2.51</td>
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<tr>
<td>Histidine</td>
<td>1.61</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.03</td>
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<tr>
<td>Tyrosine</td>
<td>0.03</td>
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MCH intake and bodyweight

Treated with MCH for 24 mo, daily food consumption did not significantly differ among the aged control (43.7 ± 3.9 g/kgbw/d), 2.25% MCH-treated (44.8 ± 3.2 g/kgbw/d) and 4.5% MCH-treated (44.4 ± 3.8 g/kgbw/d) aged groups. During the study, the intake of MCH in aged control and aged MCH-treated groups were estimated to be 0, 1.008, 1.998 g/kgbw/d, respectively. Bodyweights of rats submitted to MCH treatment were similar to those of the control rats at the beginning (controls: 93.00 ± 13.46 g; 2.25% MCH: 93.30 ± 11.45 g; 4.5% MCH: 94.00 ± 14.79g); P > 0.05) and at the end of the study (controls: 886.67 ± 137.68 g; 2.25% MCH: 843.88 ± 109.42 g; 4.5% MCH: 934.00 ± 65.05 g; P > 0.05), indicating that long-term administration of MCH did not notably affect their appetite or provoke malnutrition.

Long-term effects of MCH on collagen accumulation and collagen fiber morphology in the chronological aged skin

The dermis thickness was determined based on Mason’s trichrome stained dorsal skin section with 40 times of magnification. As shown in Figure 1A and 1B, the dermis thickness of middle-aged control rats was about twice that of aged control rats (middle-aged controls: 650.10 ± 582.98 μm; aged controls: 3340.52 ± 448.35 μm; P < 0.001). Compared with the aged control group, long-term application of MCH dose-dependently increased the dermis thickness with significance in 4.5% MCH group (4.5% MCH: 4108.23 ± 314.48 μm; P < 0.05). As a quantitative measure of total collagen, the hydroxyproline content was determined. The result in Figure 1C indicated a significant age-related decrease of hydroxyproline content in skin (middle-aged controls: 17.98 ± 3.08 μg/mg, aged controls: 12.02 ± 2.20 μg/mg, P < 0.001). The hydroxyproline content in the 4.5% MCH-treated group was 1.26-fold higher than that in the vehicle-treated aged group (4.5% MCH: 15.14 ± 2.09 μg/mg, P < 0.05). As a whole, the increased dermal thickness and total collagen content in MCH-treated chronological aged skin indicated the positive effect of MCH on collagen accumulation.

Meanwhile, we examined the effect of MCH on the morphology alteration of collagen fibers in the chronological aged skin. As shown in the Mason’s trichrome stained sections with 200 times of magnification (Figure 1A), collagen fibers in the dermis of aged vehicle control group showed weak blue staining and appeared to be more sparsely, fragmented, and disorganized versus the middle-aged control group. Compared with the aged control group, collagen fibers of the MCH-treated groups were obviously denser and more systematic with a dose-dependent tendency. Thus MCH was revealed with the inhibitory effect on collagen fragmentation in the chronological aged skin.

As a whole, the increased dermal thickness and total collagen content in MCH-treated chronological aged skin indicated the positive effect of MCH on collagen accumulation.

Long-term effects of MCH on the expressions of collagen type I and collagen type III in the chronological aged skin

Further investigations on the effects of MCH on the levels of collagen type I and collagen type III were performed by immunohistochemistry, western blot, and quantitative real-time PCR analysis, respectively.

As shown in Figure 2A, skin sections are immune stained with antibody for collagen type I and collagen type III in the area of extracellular matrix of dermis. The type I collagen fibers with dense distribution were stained strong brown whereas the type III collagen fibers were showed as faint brown with sparse distribution. Compared with the middle-aged control group, the 2 types of collagen fibers in the vehicle-aged control group
showed weaker staining and looser distribution. However, long-term MCH-treated chronological aged skin showed greater staining and denser distribution of collagen fibers.

Consistent with the immunohistochemical profile, the western blot analysis showed in Figure 2B revealed that the collagen type I and type III expression levels in the vehicle-treated aged controls significantly decreased to 37.9 ± 7.5% and 42.2 ± 8.5% of the middle-aged controls ($P < 0.001$), respectively. Compared with the vehicle-aged control group, the expression levels of collagen type I in 2.25% and 4.5% MCH-treated groups were 1.84 ± 0.36 and 1.96 ± 0.31 times of the aged control group ($P < 0.01$), respectively. Similarly, MCH administration significantly increased the expression of collagen type III in 2.25% and 4.5% MCH-treated groups to 1.38 ± 0.27 times ($P < 0.05$) and 1.92 ± 0.22 times of the aged controls ($P < 0.001$), respectively. Therefore, long-term treatment of MCH was indicated to markedly increase the expression levels of collagen type I and collagen type III in the chronological aged skin.

Next, the effects of MCH treatment on the mRNA levels of procollagen type I (COL1A2) and III (COL3A1) were revealed from the quantitative real-time PCR analysis (Figure 2C). Both the mRNA levels of COL1A2 and COL3A1 in the chronological aged skin decreased to 41.0 ± 10.1% and 47.8 ± 8.5% of the middle-aged controls, respectively ($P < 0.001$). Administration with MCH of 2.25% and 4.5% dose-dependently promoted the synthesis of type I and III procollagen in chronological aged skin. In 2.25% and 4.5% MCH-treated groups, the level of COL1A2 increased to 1.38 ± 0.2 times ($P < 0.05$) and 1.68 ± 0.29 times ($P < 0.001$) of the aged controls, and the level of COL3A1 was 1.29 ± 0.23 times ($P < 0.05$) and 1.38 ± 0.17 times ($P < 0.01$) of the aged controls, respectively.

From these results, we found that long-term oral administration of MCH could promote the expressions of collagen type I and type III proteins as well as increase the procollagen mRNA levels in chronological skin.
Long-term effects of MCH on the expressions of TGFβRII and Smad proteins in the chronological aged skin

The binding of TGF-β to TβRII forming a complex is the initial step of the TGF-β/Smad signaling cascade that regulates procollagen synthesis and extracellular matrix production in skin (Massague 1998). The results of Figure 3A revealed that the level of TβRII significantly decreased in the aged vehicle control group as compared with that of the middle-aged vehicle control group (aged controls: 50.7 ± 5.8% of middle-aged controls, P < 0.001). The level of TβRII considerably reduced in the MCH-treated aged skin as compared with that of the aged controls (2.25% MCH: 1.42 ± 0.20 times of the aged controls, P < 0.01; 4.5% MCH: 1.35 ± 0.27 times of the aged controls, P < 0.001).

As the receptor-regulated transcription factors, Smad2 and Smad3 mediate TGF-β signals when being phosphorylated (Schiller and others 2004). In our study, age-related decreases in the levels of Smad2 and Smad3 (Figure 3B) in chronological aged skin were revealed from the comparisons between the middle-aged control group and the aged control group (Smad2 of middle-aged controls: 1.36 ± 0.15 times of aged controls, P < 0.01; Smad3 of middle-aged controls: 1.20 ± 0.07 times of aged controls, P < 0.05). Although no noticeable effect on the Smad2 and Smad3 protein levels was indicated from the comparison between MCH-treated groups and the aged control group (P > 0.05), MCH showed a remarkable effect on the phosphorylation of Smad2 and Smad3. As shown in Figure 3B, the ratio of p-Smad2/Smad2 in 2.25% and 4.5% MCH-treated groups significantly increased 1.28 and 1.16 times of the aged control group (P < 0.05), respectively. Similarly, the ratio of p-Smad3/Smad3 in 2.25% and 4.5% MCH-treated groups significantly increased 1.23 and 1.18 times of the aged control group (P < 0.05), respectively.

Smad7 acts as the inhibitory Smad (I-Smad) with the activity to inhibit the signaling function of receptor-activated Smads thus interfere with the TGF-β/Smad signaling pathway. Figure 3B
showed that the protein level of Smad7 in aged control group significantly increased to 1.25 ± 0.10 times of middle-aged control group ($P < 0.01$). Smad7 protein level in the 4.5% MCH-treated group markedly decreased as compared with that of the aged control group (4.5% MCH: 82.6 ± 9.3% of aged controls, $P < 0.05$).

Long-term effects of MCH on the expressions of MMP-1 and TIMP-1 proteins in the chronological aged skin

Type I and III collagens are initially degraded by the matrix metalloproteinases-1 (MMP-1), which activity can be inhibited by the TIMP-1 (Nagase and Woessner Jr 1999). As shown in Figure 4, the expression level of MMP-1 in the aged control groups increased 2-fold versus the middle-aged control group ($P < 0.001$). Long-term oral ingestion of 4.5% MCH in diet was shown to significantly inhibit the increased MMP-1 expression level in the chronological aged skin (4.5% MCH: 67.4 ± 9.4% of aged controls, $P < 0.001$). As the specific inhibitors of MMP-1, the level of TIMP-1 showed no significant age-related difference from the comparison between the middle-aged control group and aged control group ($P > 0.05$). While 4.5% MCH treatment significantly elevated the expression level of TIMP-1 in the chronological aged skin (1.37 ± 0.19 times of aged controls, $P < 0.05$).

Long-term effects of MCH on the oxidative stress in the chronological aged skin

The oxidative stress in the chronological aged skin was assessed by the activity of SOD and the level of lipid peroxidation product (TBARS) in skin homogenates. As the Table 2 indicated, compared with the middle-aged control group, the SOD activity in the skin homogenates of aged control group significantly decreased while the TBARS level significantly increased ($P < 0.01$). The TBARS level in the chronological skin homogenates of MCH-treated groups dose-dependently decreased, with significance in 4.5% MCH group ($P < 0.05$). MCH treatment resulted in an elevation in the activity of SOD enzyme, while no statistical difference was revealed ($P > 0.05$).

Discussion

Marine proteins are likely to contain a lot of specific and potent bioactive subsequences in nutritional perspective for their competitive and aggressive living conditions (Aneiros and Garateix 2004). With various bioactive properties, MCH has gained increasing popularity as ingredients of functional foods or pharmaceuticals (Kim and Mendis 2006). To the best of our knowledge, the current study first revealed the effects of long-term oral administration of collagen hydrolysate from Chum Salmon on the chronological skin aging in vivo. Moreover, the effects of MCH on the primary molecular mechanism of collagen synthesis and degradation were elucidated.

In line with the characteristics of chronological aged skin (El-Domyati and others 2002; Varani and others 2006), the skin of aged controls was suggested with significant decreased collagen content and increased collagen fragmentation. In this study, long-term oral ingestion of MCH was found to have inhibitory effects on the age-related decreases in dermal thickness, collagen content as well as the expressions of collagen type I and III. Moreover, compared with the aged controls, the denser and more systemic collagen fibers in the dermis treated with MCH indicated the long-term oral administration of MCH might inhibit the increased degradation of collagen fibers in chronological aged skin. As we know, both the quantity and quality of collagen fibers are

Table 2—The effects of long-term oral administration of MCH on the SOD activity and TBARS level in chronological aged skin homogenate of S-D male rats.

<table>
<thead>
<tr>
<th>Group (MCH%)</th>
<th>n</th>
<th>SOD activity (U/mg protein)</th>
<th>TBARS (MDA nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle-aged control</td>
<td>10</td>
<td>128.83 ± 12.05**</td>
<td>1.76 ± 1.07**</td>
</tr>
<tr>
<td>Aged control</td>
<td>10</td>
<td>96.13 ± 24.93***</td>
<td>3.75 ± 1.57***</td>
</tr>
<tr>
<td>Aged MCH 2.25%</td>
<td>10</td>
<td>102.77 ± 21.50*</td>
<td>2.60 ± 1.17</td>
</tr>
<tr>
<td>Aged MCH 4.5%</td>
<td>10</td>
<td>104.50 ± 15.58*</td>
<td>2.27 ± 1.11*</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM, which were analyzed by one-way ANOVA test. *$P < 0.05$ and **$P < 0.01$ versus the aged control group. *$P < 0.05$ and ***$P < 0.01$ versus the middle-aged control group. (By Jiang Liang, Xinrong Pei, Zhaofeng Zhang, Nan Wang, Junbo Wang, and Yong Li.)
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determined by the balance between collagen degradation and synthesis (Lee and others 2007). In this regard, long-term MCH treatment was indicated to play an important role in the process of collagen metabolism.

The level of collagen biosynthesis can be generally reflected by the levels of type I and type III procollagen, the precursor molecules of mature collagen (Chung and others 2001). In chronological aged skin, both the number of fibroblasts and their capacity to synthesize procollagen were reduced as compared with young skin (Varani and others 2000; Chung and others 2001). In this study, the mRNA levels of procollagen type I (COL1A2) and type III (COL3A1) were shown to decrease with age, which indicate the potential of long-term oral administration of MCH to promote the expressions of type I procollagen gene (COL1A2) and type III procollagen gene (COL3A1). In line with the result, collagen extract from scallop shell was reported to increase the mRNA expression level of type I collagen in skin fibroblast cells and activate the collagen metabolism (Torita and others 2007).

It is confirmed that the production of type I and type III procollagen and other components of the dermal extracellular matrix can be primarily regulated by TGF-β, which is a family of profibrotic cytokines with the ability to exert potent stimulatory effects on collagen synthesis, extracellular matrix accumulation, and fibroblast proliferation through the cellular Smad signal transduction pathway (Fisher and others 2002). TGF-β/Smad signaling pathway is verified to directly promote the mRNA expressions of COL1A2 and COL3A1 (Chen and others 1999; Schiller and others 2004). The combination of TGF-β to the TβRII is the initial step. TβRII then phosphorylates TβRI, which can phosphorylate Smad proteins and propagate signals to the nucleus (Massague 1998). As previous study described (Rittie and Fisher 2002), TβRII protein level in this study was shown to decrease with age. It is demonstrated that transcriptional down-regulation of TβRII is primarily responsible for both the reduced TGF-β responsiveness and the impairment of TGF-β/Smad pathway, which together contribute to the reduced procollagen synthesis in skin fibroblasts (Rittie and Fisher 2002; Quan and others 2004). Therefore, we speculate that the up-regulated expression of TβRII in MCH-treated groups might resulted in the increased TGF-β responsiveness and up-regulation of type I and type III procollagen in the chronological aged skin. Furthermore, the effect of MCH on the Smad signaling was revealed in this study. It is well known that the phosphorylation of receptor-activated Smad2 and Smad3 is an initial molecular event of Smad signaling and can be prevented by I-Smad, as Smad6 and Smad7 (Rittie and Fisher 2002; Quan and others 2004). In this study, the data showed that the levels of phosphorylated Smad2 and Smad3 were elevated in all MCH-treated groups, whereas the level of Smad7 was significantly attenuated in 4.5% MCH-treated groups. When taken together, these data suggested that MCH had the potential to promote procollagen synthesis in the chronological aged skin with the mechanism involved in the activation of Smad signaling induced by the increased TGF-β responsiveness.

Moreover, except reduction of procollagen biosynthesis, increased degradation of mature collagen fibers with a concomitant increase of MMPs expression also contributes to the unbalanced homeostasis in chronologically aged skin (Kim and others 2006). Consistent with previous reports (Fisher and others 2002), the present study showed the expression of MMP-1 protein in the skin of aged control rats significantly increased as compared with the middle-aged controls. Over-expression of MMP-1 can result in the increased collagen fragmentation, which in turn promotes oxidative stress and elevates MMP-1 expression in fibroblasts (Varani and others 2006; Fisher and others 2009). As the specific inhibitor of MMP-1 activity, TIMP-1 expression in our study did not show significant age difference, which was in accordance with the result of Fisher and others (2009). The long-term administration of MCH at 4.5% concentration was suggested to significantly inhibit the expression of MMP-1 and elevate the level of TIMP-1 in the chronological aged skin. Therefore, the elevated MMP-1 protein and reduced TIMP-1 protein levels together contributed to the inhibitory effect of MCH on the degradation of collagen fibers in chronological aged skin.

In addition, MCH treatment was shown to markedly inhibit the lipid peroxidation product (TBARS) level in the aged skin homogenates rather than increase the activity of SOD, which indicated MCH itself might have the antioxidant property. Lin and Li (2006) found that the low-molecular-weight proteins and peptides, especially the fractions with molecular weights below 2000 Da, had high radical scavenging activity due to the relatively high amounts of some amino acids such as Gly, Pro, and Ala. In our study, the collagen hydrolysate rich in Gly > Glu > Pro > Hyp was
shown to be consisted of 91.5% of peptide fractions with molecular weights below 1000 Da. Thus MCH in the present study is confirmed with relative high antioxidant activity. As previous study described, the antioxidant ability of fish collagen hydrolysate is verified to play an important role in anti-UV-B induced photoaging (Zhuang and others 2009). Hence, the antioxidative activity of MCH was thought to be an important contributory factor affecting the aberrant extracellular homeostasis in the chronological aged skin. Besides that, after oral ingestion, collagen hydrolysate was verified to be degraded into peptide forms in plasma, such as Pro-Hyp and Pro-Hyp-Gly peptides. These degraded oligopeptides were demonstrated in vitro studies to function as chemotactic stimuli for fibroblasts to trigger the synthesis of new collagen fibers as well as the extracellular matrix reorganization in a protein-specific manner (Iwai and others 2005; Zague 2008). However, our data do not allow us to directly ascertain the chemotactic activity of MCH and further research is still needed to clarify the functional peptide fragments in MCH.

Conclusions

The present study demonstrated that the protective effects of long-term MCH on chronological skin aging may be due to affecting extracellular collagen homeostasis in the aged skin of S-D male rats. MCH was verified to promote collagen type I and type III synthesis through activating Smad signaling pathway with up-regulated TβRII protein level. Meanwhile, MCH inhibited the age-related increased collagen degradation through attenuating MMP-1 expression and elevating TIMP-1 expression in chronological aged skin. Moreover, the antioxidative property of MCH might play an important role during the process. All evidences earlier, taken together, lead us to propose that long-term oral administration of MCH from Chum Salmon might be a beneficial method for slowing chronological skin aging.

Acknowledgments

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