Functional roles of fish collagen peptides on bone regeneration

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Fish collagen peptides (FCP) derived from the skin, bones and scales are commercially used as a functional food or dietary supplement for hypertension and diabetes. However, there is limited evidence on the effects of FCP on the osteoblast function in contrast to evidence of the effects on wound healing, diabetes and bone regeneration, which have been obtained from animal studies. In this narrative review, we expound on the availability of FCP by basic research using osteoblasts. Low-concentration FCP upregulates the expression of osteoblast proliferation, differentiation and collagen modifying enzyme-related genes. Furthermore, it could accelerate matrix mineralization. FCP may have potential utility as a biomaterial to improve collagen quality and promote mineralization through the mitogen-activated protein kinase and Smad cascades. However, there are few clinical studies on bone regeneration in human subjects. It is desirable to be applied clinically through clinical study as soon as possible, based on the results from basic research.

Keywords: Fish collagen peptide, Osteoblast differentiation, Bone regenerative medicine

INTRODUCTION

Collagen is a major component related to extracellular matrix proteins in multicellular animals including humans, and plays an important role in maintaining a cytoskeleton. Furthermore, it has been widely used as a biomaterial (*e.g.*, as a scaffold for cell and tissue regeneration-related proteins, wound dressing, or dietary supplement)¹⁻¹⁷ because of its biocompatibility. In particular, collagen peptides, which are enzymatically hydrolyzed from gelatin, are often administered orally because they are easily digestible¹⁸, and have been reported to have a positive effect on skin, cartilage and bone¹⁹⁻³².

Tanaka *et al.* reported that the oral administration of fish scale collagen peptides to hairless mice inhibited Ultraviolet Light B (UV-B)-induced skin dehydration, reduced epidermal hyperplasia, and decreased soluble type I collagen²⁵. In addition, Cod skin-derived collagen peptides have been shown to inhibit photoaging by suppressing the expression of matrix metalloproteinases 1, 3, 9 *via* the mitogen-activated protein kinase (MAPK) pathway³³. In other words, collagen peptides have a role in protecting against skin aging. In addition, clinical trials have shown that a 6-week intake of fish collagen peptides (FCP) improved skin hydration in female volunteers^{34,35}. Kalil *et al.* reported in their clinical study that there were no side effects or systemic effects by FCP, and that the FCP-treated group showed not only better skin texture and hydration, but also improved brightness³⁶. Collagen peptides derived from marine sponge have been shown to exhibit significant antioxidant activity, and to protect cells from UV-induced apoptosis in mouse fibroblasts and human keratinocytes³⁷. Thus, they have potential

applications in the repair of damaged or photoaged skin³⁷. Zang *et al.* reported that oral administration of collagen peptides derived from Chum salmon skin promoted cutaneous wound healing and angiogenesis through upregulation of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 expression in rats, demonstrating the potential role of FCP as a healing material³⁸. Furthermore, the administration of collagen peptides from Chum salmon has also been reported to have a protective effect against early alcoholic liver injury by interfering with increased levels of total cholesterol and triacylglycerol¹⁶ and to have an accelerated healing effect on gastric ulcers¹⁵. Thus, it has been shown to be effective for wound healing of mucous membranes as well as skin. The liver is composed of hepatic lobules, which are lobular veins, interlobular arteries, and interlobular bile ducts, separated from the liver lobules by a connective tissue, Glisson's sheath. When inflammation, such as hepatitis, develops, Glisson's sheath is infiltrated by inflammatory cells and blood glutamyltranspeptidase (GTP) is known to increase³⁹. When collagen peptides are ingested, blood peptides, such as proline-hydroxyproline (Pro-Hyp) and hydroxyproline-glycine (Hyp-Gly) inhibit the cellular inflammation induced by tumor necrosis factor (TNF)- α ; it was thought that this process reduced the inflammation in a collagen peptide-treated group⁴⁰. Several other studies have reported that collagen peptides are also effective in diabetes and hypertension⁴¹⁻⁴³. Hatanaka *et al.* reported that the glycine-proline-hydroxyproline (Gly-Pro-Hyp) tripeptide is an enzyme that inactivates the hormone that lowers blood glucose levels. It has been reported that it inhibits dipeptidylpeptidase-IV activity⁴³. In a rat model of type 2 diabetes, collagen

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peptides derived from the skin of Chum salmon were reported to protect carotid artery vascular endothelial cells by lowering blood glucose levels, and were found to decrease the expression of inflammatory cytokines and adipocytokines in the liver^{44,45}. In addition, treatment with FCP has been shown to improve glucose and lipid metabolic profiles, insulin sensitivity, renal function, and hypertension management in patients with type 2 diabetes and hypertension, suggesting that FCP may have potential utility in patients with both type 2 diabetes and hypertension⁴¹. Moreover, it has reported that FCP stabilizes blood glucose levels, reduces the risk of obesity, and promotes prolonged satiety in the clinical study by patients with type 2 diabetes⁴⁶. Study using mouse 3T3-L1 preadipocytes has shown that it inhibits adipocyte differentiation through a mechanism involving transcriptional repression of main adipogenic regulators, thereby suppressing weight gain and adipogenesis⁴⁷. Angiotensin-converting enzyme (ACE) inhibitors (*e.g.*, ramipril) are known to have not only a renoprotective effect but to also reduce blood pressure, as they reduce intraglomerular pressure by the dilation of the renal tubules⁴⁸. Collagen peptides have been shown to have ACE inhibitory activity⁴⁹ and may therefore contribute to the suppression of elevated blood pressure. Articular cartilage damage is one of the most common musculoskeletal disorders we encounter, and affects our quality of life. It has been suggested that collagen peptides extracted from the skin of deep-sea fish such as cod, haddock, and pollock may promote chondrogenic differentiation of primary adipose-derived stromal cells⁵⁰. It was also reported that oral administration of FCP and glucosamine suppressed cartilage degradation in rabbits with osteoarthritis⁵¹. In line with these findings, FCP was shown to promote the chondrogenic differentiation of human mesenchymal stem cells⁵². In clinical studies, FCP has been proven to promote the synthesis of cartilage matrix and reduce pain in patients with osteoarthritis, making it a promising candidate for the treatment of human osteoarthritis^{46,53-55}. Collagen peptides are thus cryptic peptides with a variety of bioactivities⁵⁶; however, the detailed mechanisms underlying their effects in these diseases are unclear.

Unlike the organs associated with the above-mentioned diseases, bones and teeth are hard tissues composed of inorganic substances (*e.g.*, calcium, magnesium, and phosphorus), organic substances (*e.g.*, type I collagen), and non-collagenous proteins (*e.g.*, osteocalcin and osteonectin)⁵⁷⁻⁶³. Based on its structural style, it is often compared to a reinforced concrete building. The reinforcing steel corresponds to collagen, while concrete corresponds to inorganic substances, such as calcium and phosphorus. Collagen provides not only the framework for bones, but also a source of bone flexibility. Osteoporosis has been known to be mainly caused by a decrease in bone density. However, in recent years, it has become clear that bone quality also plays a significant role in osteoporosis⁶⁴⁻⁶⁷. Collagen is the critical factor for bone quality. At the National Institutes of Health (NIH) Consensus Conference in

2000, the definition of osteoporosis was revised from the conventional approach, to the following definition, which focuses on bone mineral density: “a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture”. In brief, both bone density and bone quality are involved in bone strength. Thus, the osteogenic effect of collagen peptides needs to be evaluated from the viewpoints of both mineralization-related markers and collagen, which serves as a three-dimensional template for mineralization.

In this paper, we review the effects of FCP on bone regeneration in terms of “characterization of collagen peptides” and “mineralization-related markers and collagen quantity and quality” and provide a perspective on the role of FCP in the field of bone regeneration.

EFFECT OF MOLECULAR WEIGHT OF COLLAGEN PEPTIDES ON OSTEOGENESIS

Several studies have explored the molecular weight of bioactive collagen peptides^{68,69}. It has been reported that rats treated with orally administered collagen peptides with a molecular weight of less than 2 kDa derived from squid skin showed excellent anti-ACE activity⁶⁸. In addition, Kim *et al.* found that collagen peptides with molecular weights of 1.5–4.5 kDa derived from Alaska cod skin showed excellent antioxidant activity and could be used as natural antioxidants by *in vitro* systems⁶⁹.

Shimizu *et al.* used Caco-2 cells to investigate intestinal permeability to molecular weight size-classified FCP⁷⁰. They revealed that the smallest sized FCP crossed the Caco-2 monolayer most efficiently and that permeability was dependent on peptide size. However, because orally ingested functional peptides may be subjected to digestive degradation in the intestinal tract and/or deactivated by chemical changes, and because some physiological functional components may not have an efficient absorption and uptake mechanism in the intestinal tract, it is difficult to determine whether the effects found by *in vitro* systems using cultured cell responses will necessarily be effected by oral ingestion. On the other hand, few clinical studies have reported the optimal molecular weight range for patients with osteoporosis or arthritis, and there have been no detailed basic research-based reports on the mechanism. Liu *et al.* reported that 1,370 and 7,747 Da of bovine bone-derived collagen peptides promoted the expression of alkaline phosphatase (ALP) in MC3T3-E1 cells, while 878 Da of collagen peptides had the opposite effect⁷¹. This result indicates that the effect of ALP is related to the molecular weight distribution of collagen peptides. In a previous study, we examined the expression of mineralization-related markers in NOS-1 cells, which is a human osteosarcoma-derived osteoblast, using FCP of ~2 kDa to ~8 kDa in molecular weight, and found that the ALP activity on day 3 of culture and the protein expressions of osteocalcin (OC), osteopontin (OP) and integrin (ITG) were significantly upregulated in an FCP-supplemented group on day 7 of

culture⁷²⁾. Furthermore, the gene expressions of the type I collagen $\alpha 2$ chain, lysyl hydroxylase (LH) 1, 2, 3 and lysyl oxidase-like (LOXL) 2, 3, 4 and glycosyltransferase 25 domain 1 (GLT25D1), which are collagen cross-linking-related enzymes, were enhanced on day 3 of MC3T3-E1 cell culture⁷³⁾. FCP also increased collagen synthesis by approximately 15% on day 14 of culture. In addition, the expression of hydroxylysine (Hyl), which is the source of collagen cross-linking, was increased by 25%. They resulted in an approximately 77% increase in the formation of pyridinoline (Pyr), a mature cross-link that exists predominantly in bone⁷³⁾. Then, alizarin red S staining on day 21 of culture revealed significantly more minerals in the FCP-supplemented group in comparison to the control group⁷³⁾. The FCP powder was further classified into three fractions of approximately 2, 2.5 and 3 kDa by molecular sieve chromatography on the basis of the chromatogram analyzed by high-performance liquid chromatography. When MC3T3-E1 cells were treated with fractionated FCP for 3 days, they proved a significant enhancement of the LH1 gene expression by approximately 17-fold (in comparison to the control group) in cells treated with 3 kDa of FCP and approximately 13-fold in cells treated with 2 kDa of FCP. As for LH2, the gene expression was enhanced by approximately 2-fold in cells treated with 2 kDa of FCP, although the difference was not statistically significant. The gene expression of lysyl oxidase (LOX) was approximately 8-fold higher than that of control group at the 2 kDa of FCP-supplemented group.

Alizarin red S staining also confirmed that MC3T3-E1 cells cultured in FCP-containing medium with a molecular weight of approximately 2.8 kDa for 21 days showed more minerals than the same cells cultured in the maintenance medium (unpublished data). These results suggest that the physiological cross-linking of collagen formed actively through collagen cross-linking-related enzymes (e.g., LH1, 2 and LOX) by 2–3 kDa of FCP might have resulted in the formation of tenacious collagen fibers, and a three-dimensional collagen template suitable for matrix mineralization promoted mineral deposition and growth.

EFFECT OF AMINO ACID SEQUENCES OF COLLAGEN PEPTIDES ON OSTEOGENESIS

Hydroxyproline, the main component of collagen, is responsible for the stability of collagen along with proline. In general, it accounts for approximately 10% of the total amino acids in mammalian type I collagen. On the other hand, the hydroxyproline content of fish collagen is approximately half that of mammalian collagen^{73,74)}. It makes the triple helix stability of fish collagen less stable in comparison to mammalian collagen^{75,76)}. Furthermore, this property leads to a lower denaturation temperature of fish collagen, while leading to better digestion and absorption than mammalian collagen.

How does the amino acid sequence of collagen peptides affect osteogenesis? Table 1⁷⁷⁻⁸⁴⁾ lists some reports on the effect of the amino acid sequences of

Table 1 The effect of amino acid sequence on collagen peptide for osteogenesis

Amino acid sequence	Effect on cell differentiation and osteogenesis	Reference No.
RGD motifs (arginine-glycine-aspartic acid)	RGD motifs induce focal adhesion through integrin binding.	77)
	RGD motifs initiate α_v integrin signaling in osteoblasts.	78)
DGEA peptides (aspartic acid-glycine-glutamic acid-alanine)	DGEA peptides cause the expression of osteogenic protein markers, e.g. collagen type 1, osteopontin, alkaline phosphatase, osteocalcin and dentin matrix acidic phosphoprotein 1.	79)
	E7(heptaglutamate)-DGEA-coupled hydroxyapatite enhances the adhesion and osteoblastic differentiation of mesenchymal stem cells, and increases new bone formation in rat tibiae.	80)
	DGEA peptides are involved in type 1 collagen- $\alpha 2\beta 1$ integrin interaction on bone marrow cells.	81)
Pro-Hyp (proline-hydroxyproline)	During osteoblast differentiation, Pro-Hyp mediates Runx2 activity though directly binding Foxg1, which has been shown to play a role in the development of the brain and telencephalon, and increases Runx2 expression.	82)
	Pro-Hyp has a positive effect on osteoblastic differentiation through upregulation of Runx2, osterix, and collagen type 1 $\alpha 1$ chain gene expression.	83)
Lys-Ser-Ala (lysine-serine-alanine)	Lys-Ser-Ala promotes cell proliferation, alkaline phosphatase activity, mineral deposition, and expressions of osteoblastic differentiation-related gene such as alkaline phosphatase, osteopontin and osteocalcin in MC3T3-E1 cells.	84)

collagen peptides on osteogenesis. The RGD motif (arginine-glycine-aspartic acid) is a cell adhesion active sequence common to many cell adhesion proteins and is present in dozens of proteins (e.g., fibronectin, OP and laminin) as well as collagen. Staatz *et al.* found that $\alpha 2\beta 1$ ITG bound to a site within an $\alpha 1(I)$ -CB3 fragment in type I collagen⁷⁷. Subsequently, D'Alonzo *et al.* found that RGD peptide mimetics initiated α_v ITG signal transduction in MC3T3-E1 cells and that OP and α_v ITG diversely regulated the expression of collagenase-3 and OC during osteoblast differentiation⁷⁸. The DGEA peptide (aspartic acid-glycine-glutamic acid-alanine) of type I collagen was revealed to induce osteoblast differentiation⁷⁹ and it was found that when using heptaglutamate (E7), which is the hydroxyapatite (HA)-binding domain, HA coupled with E7-DGEA promoted mesenchymal stem cell adhesion and osteoblast differentiation and increased new bone⁸⁰. DGEA peptides may not necessarily have a positive effect on osteogenesis, as it has been reported that the addition of DGEA peptides suppresses the expression of the osteoblast phenotype in bone marrow cells by interfering with the interaction between type I collagen and $\alpha 2\beta 1$ ITG⁸¹.

Pro-Hyp has been shown to mediate Runx2 activity and increase the Runt-related transcription factor (Runx2) expression by directly binding to Forkhead Box G1 (Foxg1) recombinant protein during osteoblast differentiation⁸². Although Foxg1 appears to interact with Runx2 in the absence of Pro-Hyp, Pro-Hyp binds directly to Foxg1 protein in the presence of Pro-Hyp, causing a conformational change in Foxg1 protein and splitting the interaction between Foxg1 and Runx2. Thus, it seems to lead to the increased expression of Runx2⁸². Another study also found that Pro-Hyp did not effect MC3T3-E1 cell proliferation or matrix mineralization, but significantly increased the ALP activity and gene expression of Runx2, osterix and type I collagen $\alpha 1$ chains⁸³. These results demonstrated that Pro-Hyp promotes osteoblast differentiation. Thus, there are frequent reports on osteoblast differentiation and matrix mineralization of peptides containing the RGD motif, which is an active sequence associated with cell adhesion, the DGEA peptide which interacts with the cell adhesion molecule ITG, as well as Hyp, which is an amino acid specific to collagen. However, lysine-serine-alanine (Lys-Ser-Ala) has also been reported to positively influence osteoblast differentiation⁸⁴. Lys-Ser-Ala, purified by liquid chromatography, had a high affinity for the core interface residues of the bone morphogenetic protein (BMP) receptor and induced phosphorylation of MAPKs, including p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase, as well as Smads. This might have promoted cell proliferation, ALP activity, mineral deposition and the expression of osteoblast differentiation markers in MC3T3-E1 cells.

EFFECTS OF COLLAGEN PEPTIDES ON COLLAGEN QUALITY AND MINERALIZATION

It has been reported that type I collagen extracted from tilapia (*Oreochromis niloticus*) scales improves cell viability and adhesion as well as ALP activity, and promotes matrix mineralization by increasing gene expression of bone sialoprotein in rat odontoblastic cells⁸⁵. It was also revealed that FCP promoted the survival of human primary periodontal ligament cells and upregulated the expression of osteogenesis-related proteins through the ERK signaling pathway, indicating that FCP might be a promising bioactive component for alveolar bone regenerative material⁸⁶.

Osteoblasts synthesize a collagen matrix, which itself regulates the differentiation of precursor cells into mature osteoblasts. LOX and LH, which are involved in the formation of collagen cross-links synthesized by osteoblasts, also actively contribute to mature collagen formation⁸⁷. Therefore, latylogens such as β -aminopropionitrile (β APN), which suppresses LOX activity, inhibit stable matrix formation. It is reported that the collagen matrix synthesized by MC3T3-E1 cells treated with β APN for 1 week significantly reduced dehydro-dihydroxylysinonorleucine (deH-DHLNL) and Pyr, which are the main cross-links of bone collagen, in comparison to control cultures⁸⁸. In addition, inhibition of the LOX expression by β APN significantly downregulated the OC mRNA expression to approximately 75%, suggesting that β APN treatment not only inhibits the formation of collagen cross-linking, but also affects the activity and expression of osteoblasts⁸⁸. In our study, as mentioned above, FCP treatment significantly increased the formation of Pyr, which is a stable reductive cross-link, along with the amount of collagen synthesis in MC3T3-E1 cells, which had a positive effect on the expression of mineralization-related proteins, such as OC, OP and ITG, resulting in accelerated mineralization^{72,73}. In addition, we have also confirmed that the filling of the FCP into the bone cavity formed in the mandible of rats accelerated osteogenic repair with minimal inflammatory cell infiltration in comparison to a control group that had no filling of FCP⁸⁹.

The effect of FCP on matrix mineralization in the presence of collagen with suppressed physiological cross-linking formation is shown in Fig. 1. In order to synthesize collagen with suppressed physiological cross-linking formation, MC3T3-E1 cells were treated with minoxidil (Mx), a specific inhibitor of LH. The group cultured in Mx-containing FCP-free medium showed little or no mineral formation on day 21 of culture, whereas a small amount of mineral deposition was observed in the group cultured in Mx- and FCP-containing medium, with the group cultured on Mx-free FCP-containing medium showing the greatest amount of mineral deposition. These results indicate that LH positively contributes to mineralization, while FCP may have the ability to improve mineralization in the presence of vulnerable collagen.

Considering our previous studies and other reports,

Fig. 1 The effects of minoxidil, which is an inhibitor of lysyl hydroxylase, and FCP on matrix mineralization in MC3T3-E1 cell culture system at 21 days. The cultures were stained with alizarin red S to visualize mineralized nodules. (a) The group cultured with 100 µM minoxidil (Mx) and 0.2% FCP-free medium. (b) The group cultured with Mx-containing and FCP-free medium. (c) The group cultured with Mx-free and FCP-containing medium. (d) The group cultured with Mx- and FCP-containing medium.

Fig. 2 The osteoblast differentiation and collagen cross-linking-related markers promoted by FCP.

MAPK: mitogen-activated protein kinase, LH: lysyl hydroxylase, GLT25D1: glycosyltransferase 25 domain 1, Col1a1,2: type I collagen α 1 chains and 2 chain, ALP: alkaline phosphatase, OP: osteopontin, OC: osteocalcin.

it is presumed that natural functional collagen peptides derived from fish skin, bones and scales may actively induce osteoblast differentiation and improve collagen quality by enhancing the expression of mineralization-related markers and collagen cross-linking-related enzyme gene expression through the Smad and ERK/MAPK cascades, resulting in matrix mineralization (Fig. 2).

On the other hand, as mentioned earlier, our previous studies proved that the gene expressions of LH3 and GLT25D1 in MC3T3-E1 cells were enhanced by FCP⁷³⁾. It has been reported that LH3 functions mainly as a glucosyltransferase, while GLT25D1 does as a galactosyltransferase for type I collagen in osteoblast culture systems⁹⁰⁾. It has also been suggested that when

LH3-mediated glucosylation occurs at a specific molecular site, *e.g.* residue 87, it is involved in the formed collagen fiber's diameter and the maturation of cross-linking⁹¹⁾. Further studies are required to determine whether the degree of collagen glycosylation in the FCP-treated group is actually increased at the protein level, and if so to investigate the relationship between glycosylation and matrix mineralization.

CONCLUSIONS AND FUTURE PROSPECTS

In the past decade, tremendous progress has been made toward the regeneration of dentin, dental pulp and periodontal tissues including alveolar bone and periodontal ligament in the field of dental tissue

engineering, giving rise to the new field of regenerative dentistry⁹²⁻⁹⁷. FCP promotes matrix mineralization by positive effects on collagen synthesis and quality in osteoblasts. Namely, it means the availability to the regeneration of dentin and alveolar bone, which are composed of organic (collagen) and inorganic (minerals) matters in the field of dentistry. It also has potential as a safe bone regeneration agent that can contribute to human health through the effective use of fish by-products, which are originally marine waste^{98,99}.

However, few clinical studies on human subjects and animal studies have yet been conducted to evaluate the bone regeneration promoting effects of FCP. In order to properly evaluate the therapeutic effects of FCP in many medical fields including the field of bone regeneration, further comprehensive studies based on the results from basic research are necessary. And clinical application of FCP as soon as possible is desirable in other medical fields as well as the field of dentistry.

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