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Collagen Biosynthesis in Osteoblast Cells Treated with Fish Collagen Peptides

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Abstract

Collagen is one of the most widely used biomaterials in tissue engineering and regenerative medicine. To investigate the effect of fish collagen peptides on the formation of collagen proteins by mature osteoblast cells, human osteoblast cells were treated with collagen peptide in both time and dose dependent manner. The stimulatory effect of collagen peptide was confirmed by western blot and immunocytochemistry analysis. The results clearly indicate that the presence of fish collagen peptides led to a dose dependent increase in collagen synthesis by osteoblast cells upto a concentration of 0.6 mg ml^{-1}. However native collagen or collagen free protein hydrolysate failed to stimulate the production of collagen in osteoblast cells. The results show a stimulatory effect of fish collagen peptides on the collagen biosynthesis in osteoblast cells and suggest a possible mechanism for the regulation of collagen turnover in joint tissues.

Keywords: Fish collagen peptides, osteoblast cells, collagen quantification, western blotting, immunocytochemistry

Introduction

Collagen peptides have been used therapeutically as a dietary supplement to improve conditions of joints (Moskowitz, 2000). Collagen hydrolysates are safer compounds with less overall toxicity that could provide, a greater symptomtic relief than pharmaceutical drugs. Oral administration of collagen hydrolysate was demonstrated to reduce bone loss by increasing the quantity of type 1 collagen and proteoglycan in the bone matrix of ovariectomized (OVX) rats (Jackix et al., 2010). Collagen hydrolysate is absorbed in its high-molecular weight form, containing peptides of 2.5-15 kDa (Oesser et al., 1999). Experimental studies have suggested that some orally given collagen-derived peptides are absorbed intact in the intestine. Subsequently, these peptides would accumulate preferably in cartilage, where they may finally stimulate cartilage metabolism (Oesser et al., 1999). Evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes (Oesser et al., 2003). Oral administration of collagen hydrolysates would provide high levels of glycine and proline, two amino acids essential for the stability and regeneration of cartilage (Nomura et al., 2005). The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific peptides on gene expression and function of chondrocytes (Walrand et al., 2008). Osteoblasts are cells responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodelling. Osteoblasts form a closely packed sheet on the surface of the bone, from which cellular processes extend through the developing bone (Cooper, 2000; Spreafico, 2006).

An in vitro study was designed to investigate the biological effects of fish collagen peptide (FCP) on human osteoblast cell lines (HOS). To elucidate this novel function regarding collagen synthesis, we treated human osteoblast cells with collagen peptide in both time and dose dependent manner followed by measurements of biosynthesis and secretion of type 1 collagen. MTT assay was done to check the cytotoxic effect of FCP on the growth of cells. During treatment with FCP, collagen synthesis is quantitatively screened in the culture cells by Sirius red staining, western blot analysis and immuno fluorescence analysis.

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Materials and Methods

An osteoblastic cell line derived from a human osteosarcoma, MG-63, was used in the present study. Cell viability was assessed using the MTT colorimetric assay according to ATCC protocols. Effect of Fish Collagen Peptides (FCP) developed from the skin of *Epinephilus malabaricus* at the Biochemistry and Nutrition Division of ICAR-CIFT was evaluated in the study.

FCP treatment and monitoring of collagen expression was done according to ATCC protocols. The cells were treated with different concentrations of FCP for different time periods (6, 12 and 24 h). After incubation, the cells were washed with 1 mL of phosphate buffered saline and collected by centrifuging at 2 800 g for 5 min at 4°C. The cells in each group were lysed in a lysis buffer and then they were sonicated with a cell disrupter for 1 min in ice cold water. The cell lysate was collected by centrifugation at 22 000 g for 10 min at 4°C and stored at -80°C till evaluation. Collagen was quantified as described by Peterszegi et al., 2008. The cell lysate containing collagen proteins obtained after FCP treatment was subjected to a Western blot analysis according to the method of Towbin et al., 1979. The protein concentrations in each group were determined by Bradford assay (Bradford, 1976).

For confocal imaging, the cells were plated at about 50% confluence on a 4-chamber glass slide and incubated in 15% FBS medium for 1 h at 37°C. While still in growth media, 500 µL medium and FCP was removed and added at 0.3 mg ml^{-1} to 1.0 mg ml^{-1} concentrations to the appropriate chambers for 24 h. The cells were fixed by adding 0.5 mL of 4% paraformaldehyde and then incubated for 30 min in 0.5 mL of 1% BSA. All remaining steps were performed in the dark to protect fluorescent markers. After washing, the cells were incubated with primary antibody (Anti-Collagen 1 antibody), for 12 h at 4°C. An FITC-conjugated goat polyclonal to rabbit IgG was used at dilution at 1/160 as secondary antibody. Immunofluorescence was visualized using Nikon AIR confocal imaging system and Andor Revolution XD Spinning Disc Microscope with and orixon 897 EMCCD cameras.

The results are expressed as mean ± SE from n=3 observations. The findings were also analyzed for determining significance of difference by ANOVA test followed by pair-wise comparison of various group by LSD. The differences among groups were considered to be significant at $p<0.05$. The analysis was carried out by using SAS system version 9.1 (SAS Institute Inc., Cary, NC, USA)

Results and Discussion

MTT assay to assess reduction in cell viability revealed that there was a significant retardation in the proliferation of cells (Table 1) at FCP above 2.0 mg ml^{-1} which affected the growth of cells.

Table 1. MTT assay

Concentration of FCP(µg ml^{-1})	% inhibition on growth of cells
0.0	09.23±0.35
0.2	09.41±0.57
0.4	09.51±0.42
0.5	10.15±0.23
0.6	12.56±0.47
0.8	12.51±0.53
1.0	14.54±0.38
1.5	21.21±0.40
2.0	30.51±0.63*
5.0	58.32±0.74*
10.0	60.51±0.29*

Values are the average of three separate experiments in quadruplicate and are expressed as mean ± SD

*$p < 0.05$

Fig. 1 shows that the treatment of cultured osteoblast cells with 0.6 mg ml^{-1} FCP over a culture period of 24 h induced a marked increase in collagen secretion into the culture medium when compared to the control cells which did not receive FCP supplementation. Oesser et al. (1999) reported that chondrocytes treated with collagen hydrolysate at concentrations ranging from 0.5 mg ml^{-1} to 5 mg ml^{-1} secreted a maximum of 2.2 fold more collagen than the untreated cells.

From western blot analysis, two immuno reactive bands were detected using anti collagen antibody (Fig. 2). The two bands obtained correspond to the α and β chains of type 1 collagen. Expression levels of type 1 collagen, from cell lysate of FCP treated cells, showed that there was dose dependent increase in type 1 collagen synthesis. Similar results were obtained by Oesser et al. (1999) using bovine chondrocytes. Results were expressed in arbitrary

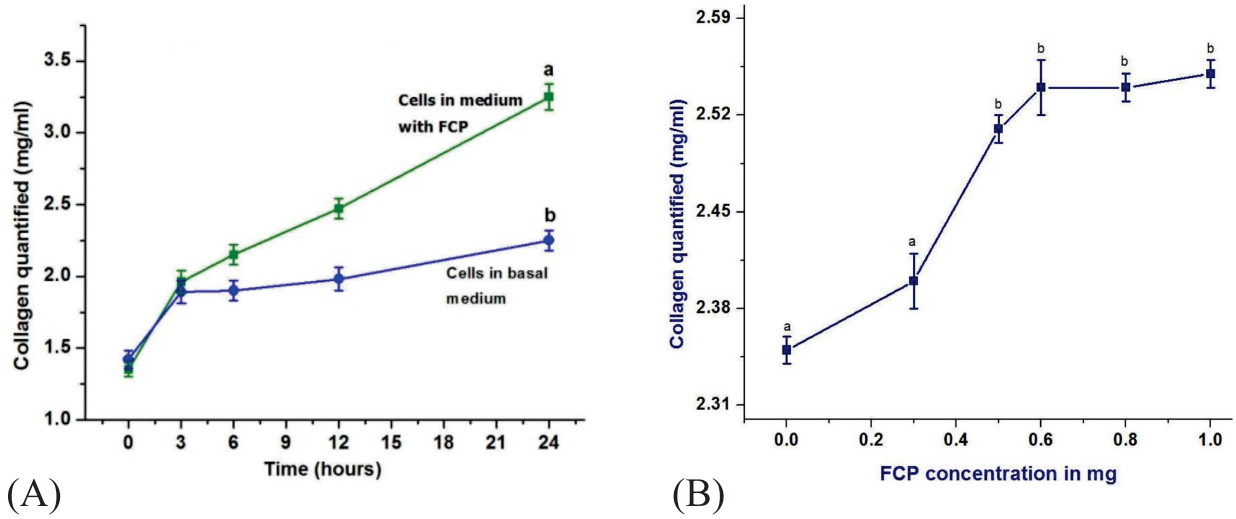

Fig. 1. Collagen quantified (A) at different time intervals at 0.6 mg ml^{-1} & and (B) at different FCP concentrations through Sirius red staining. The results were expressed as mean ± SE. Data were analyzed by one way ANOVA followed by post-hoc procedure using SAS 9.3

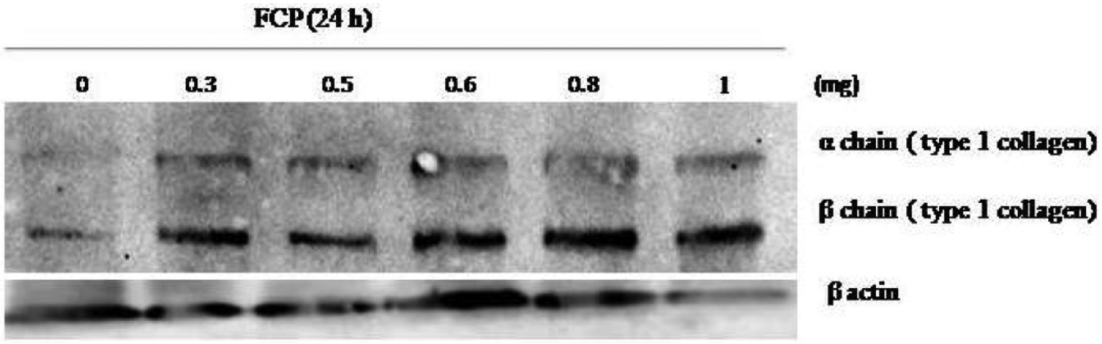

Fig. 2. Western blotting using antibodies against type 1 collagen

densitometric units normalized for the expression of β actin in each sample (mean±SD of five separate experiments).

The stimulation of type 1 collagen synthesis was fluorescently visualized by means of immunocytochemistry (Fig. 3). After 24 h of incubation, osteoblast cultures treated with FCP 0.5, 0.6 and 0.8 mg mL^{-1} concentrations significantly deposited tight nets of collagen fibres pericellularly, where as in normal cultures the measurable amount of cell associated type 1 collagen was considerably reduced. Recently, it was reported that treatment with collagen hydrolysate from porcine skin gelatin significantly increased the collagen content and expression of the Col1α1 gene through phosphorylation of ERK (Kim et al., 2013). Ishida et al. (1981) used the immuno-cytochemical techniques for showing the increased expression of type 1 collagen by cells upon induction with synoviolin. Oesser et al. (1999) in his investigations examined the stimulatory effect of bovine collagen hydrolysate in the metabolism of chondrocytes using immunocytochemical techniques. As bone health supplements, bovine collagen peptide compounds are thought to stimulate increased synthesis of collagen (Yamada et al. 2013). Schadow et al. (2010) demonstrated that collagen hydrolysates from various sources differ significantly with respect to both their chemical composition of oligopeptides representing collagen

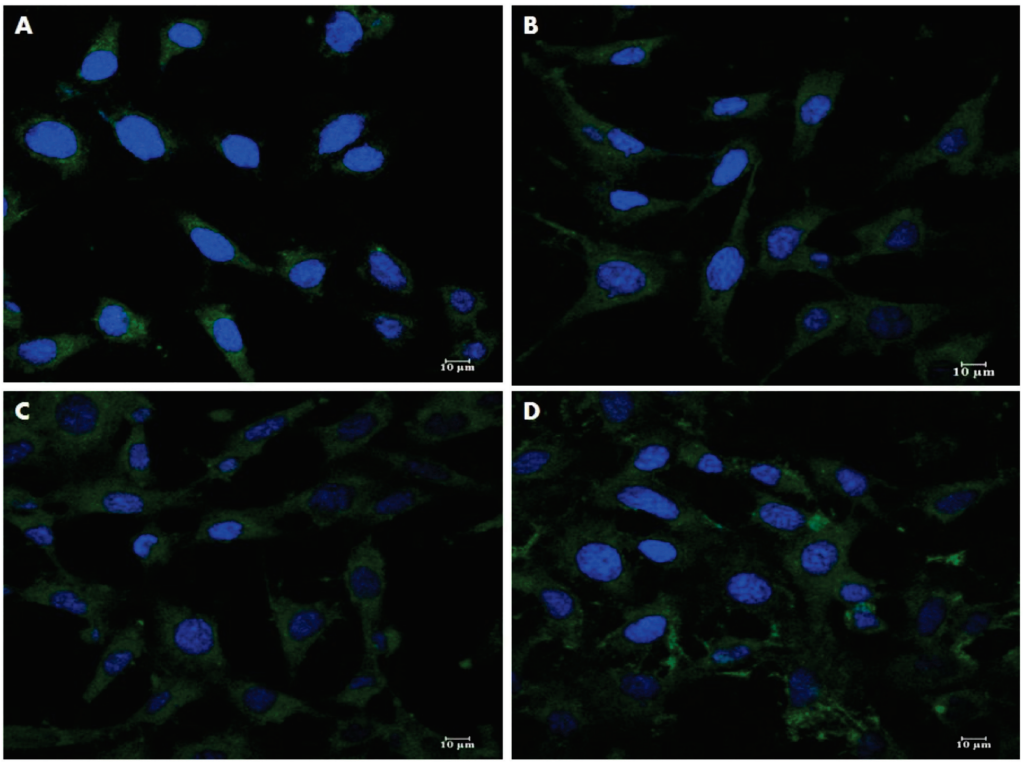

Fig. 3. Immunocytochemical visualization of type 1 collagen pericellularly. A, is control cultures grown in media without FCP; B, C and D are cultured in media with 0.5, 0.6, 0.8 mg mL^{-1} FCP respectively. The results were derived from 3 independent experiments. Scale bar = 10 µm

fragments as well as their effect on human articular cartilage.

It is concluded from the study that enzymatically hydrolyzed collagen preparations might contain therapeutically useful peptides which can stimulate collagen biosynthesis in osteoblast cell lines. The biomedical properties of FCP have to be studied thoroughly in clinical trials before being applied as a safe and effective drug in human beings.

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