Dental Materials Journal 2013; 32(2): 233–240

Early gene and protein expression associated with osteoblast differentiation in response to fish collagen peptides powder

Shizuka YAMADA[1], Yuu YOSHIZAWA[1], Atsushi KAWAKUBO[1,2], Takeshi IKEDA[1], Kajiro YANAGIGUCHI[1] and Yoshihiko HAYASHI[1]

[1] *Department of Cariology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan*
[2] *Motohara Dental Clinic, Motohara-machi 13-15-1F, Nagasaki 852-8133, Japan*
Corresponding author, Shizuka YAMADA; E-mail: shiduka@nagasaki-u.ac.jp

This study was designed to investigate the biological effects of fish collagen peptide (FCP) on human osteoblasts. Human osteoblasts were treated with 0.1% FCP, which was the optimal concentration confirmed by the increase in alkaline phosphatase activity. After one, three, five and seven days of culture, the number of FCP-treated cells increased significantly compared with untreated cells. In a real-time PCR analysis, the expression of osteocalcin, osteopontin, BMP-2 and integrin β3 mRNAs in FCP-treated cells showed increases compared with untreated cells after three days of culture. After seven days of culture, the expression levels of osteopontin and integrin β3 were still higher in the FCP-treated cells than in untreated cells. The production of osteocalcin, osteopontin and integrin β3 proteins in FCP-treated cells also showed increases after seven days of culture. Furthermore, FCP accelerated matrix mineralization in the cultures. The present study indicates the potential utility of FCP as a biomaterial.

Keywords: Fish collagen peptide, Osteoblast differentiation, Alkaline phosphatase activity, Real-time PCR, Western blot analysis

INTRODUCTION

Collagen is a major component of the extracellular matrix in multicellular organisms, including humans, and is an important component of ligaments, cartilage and bone. It accounts for about 30% of the total proteins in the body. Type I collagen is the most common type of collagen, and is abundant in bone. Therefore, this protein has been generally recognized as a safe material and is widely utilized in a variety of products ranging from health food (supplements) to cosmetics. It was reported in a double-blind trial that the ingestion of 10 g of pharmaceutical-grade collagen hydrolysate daily for two months reduced the pain by half in patients with osteoarthritis of the knee or hip in comparison to a placebo[1,2]. Furthermore, Koyama *et al.* showed that the bone mineral density of the femora in normal mice increased in response to a diet supplemented with 4% gelatin in comparison to a 10% casein diet[3]. The purified mineralization-inducing factor in conditioned medium from preosteoblastic cells contained type I collagen, which suggests that collagen serves, at least in part, as differentiation-inducing agent for mineralization[4]. The clinical use of materials derived from bovines is prohibited at present due to the finding of Bovine Spongiform Encephalopathy (BSE) in the United Kingdom in the late 1980's. Therefore, fish collagen has recently been used for products such as a health food supplements for osteoarthritis, osteoporosis and rheumatoid arthritis.

Fish collagen is extracted and purified from the skin, bones, swim bladder and scales of various fish species. Active oxygen is eliminated from the body, and cholesterol in the blood decreases after the consumption of the methionine-rich fish collagen. The stability of the triple helix in fish collagen is reduced compared to mammalian collagen, because fish collagen has a lower content of hydroxyproline[5,6]. Therefore, its digestive absorption is thought to be superior to that of mammalian collagen. Importantly, its denaturation temperature is lower than that of mammalian collagen.

As fish collagen is difficult to dissolve in a neutral pH medium, the soluble type of collagen, fish collagen peptide (FCP) must be used in *in vitro* study. Furthermore, there have been no reports about gene expression using FCP in relation to biological mineralization *in vitro*. This study was designed to investigate the subcellular effects of FCP on human cultured osteoblasts.

MATERIALS AND METHODS

Preparation of fish collagen peptide solution

The FCP powder (Marine matrix®) used in this study was supplied by Yaizu Suisankagakukougyou Co., Ltd. (Shizuoka, Japan). This FCP was produced by pepsin digestion of the bone and skin from cods caught in the sea near Shizuoka (personal communication from company). The molecular weight of the FCP used in this study was 500–10,000 Da and the majority of the peptides were about 3,000 Da. The FCP consisted of a little lower concentration of hydroxyproline (5.14% of the total amino acids) than that usually present in bovine collagen (8.70% of the total amino acids), whereas the methionine content (1.04% of the total amino acids) was almost equivalent to that of bovine collagen (1.70% of the total amino acids) as determined by an amino acid analysis with high performance liquid chromatography (HPLC) (personal communication from Prof. Mitsuo Yamauchi). A 5% (w/v) solution of the FCP was prepared

The first two authors contributed equally to this work.
Received Jul 17, 2012: Accepted Dec 13, 2012
doi:10.4012/dmj.2012-188 JOI JST.JSTAGE/dmj/2012-188

by dissolving powdered collagen in α-modified essential medium (α-MEM) (Invitrogen, Carlsbad, CA, USA), and it was filtered using a 0.2 μm filter.

Casein peptide was purchased from Nacalai tesque (Kyoto, Japan) to investigate the effects of the amino acid itself on osteoblast proliferation and differentiation as control amino acids. It was also prepared by the above-mentioned processes.

Osteoblast culture

An osteoblastic cell line derived from a human osteosarcoma, NOS-1, was used in the present study. The cells were seeded in 60 mm culture dishes at a density of 2×10^5 cells. They were then cultured for seven days in α-MEM supplemented with 10% fetal bovine serum (CELLect® GOLD, MP Biomedicals Inc., Solon, OH, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37ºC. The medium was replaced every three days.

Alkaline phosphatase activity assay

The NOS-1 cells were seeded in 60 mm culture dishes at a density of 2×10^5 cells, and were cultured under the same conditions stated above. In the FCP-treated group, a 5% FCP solution was added to α-MEM such that the total concentrations were 0.0005, 0.005, 0.05, 0.1 and 0.5% (w/v). As a control, culture dishes filled with α-MEM without the FCP solution were also placed in the incubator. After three days of incubation, the cells were rinsed twice with phosphate-buffered saline (PBS) (−) and scraped off the dish with NUNC™ Cell Scrapers (Thermo Fisher Scientific International, Rochester, NY, USA). After that, they were centrifuged at 1,000 rpm for 10 min and the supernatant was discarded. Next, 0.01N TES saline (pH 7.4) was added. The cells were then sonicated with a cell disrupter (VP-30S, Taitec, Saitama, Japan) for 1 min in ice-cold water. The alkaline phosphatase (ALP) activity was determined by a technique using ρ-nitrophenyl phosphate as a substrate. The micro-Lowry method was used to determine the protein concentration[7]. The specific activity was expressed as the micromoles of ρ-nitrophenol produced per minute per milligram of protein. The total concentration of FCP solution that resulted in the highest activity was considered to be the optimal concentration for NOS-1 cells.

The cells in both the FCP and casein peptide (CP) groups were incubated with the optimal concentration determined by the ALP activity assay. After three days of culture, the cells were retrieved using a cell scraper. The ALP activity and protein concentration were measured using the above-mentioned procedures.

Cell proliferation study

NOS-1 cells were seeded in 60 mm culture dishes at a density of 2×10^5 cells, and were cultured with or without FCP and CP. Cells in the FCP and CP groups were incubated with the optimal concentration determined by the ALP activity assay. After one, three, five and seven days of culture, the number of viable cells, which were not stained by trypan blue, was counted using a hemocytometer under a phase-contrast microscope. Digital microphotographs were analyzed with Scion image software in order to obtain the cell's area per focal field investigated.

PicoGreen® DNA assay

NOS-1 cells were seeded in 48-well plates at a density of 2×10^5 cells and were cultured under the same conditions stated above. The cells in the FCP and CP groups were incubated with the optimal concentration determined by the ALP activity assay. After one, three, five and seven days of culture, the cells in each group were retrieved. The DNA content was measured using the Quant-iT™ PicoGreen® dsDNA Reagent and kit (P7589, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, equal quantities of the diluted sample and PicoGreen dye were added to a 96-well plate and incubated in the dark at room temperature for 5 min. A standard curve based on known concentrations of DNA was utilized for the calculations. Both the standard and experimental samples were read on a fluorescence plate reader (DTX 800 Multimode Detector, Beckman Coulter, Boulevard Brea, CA, USA) at 485/535 nm (excitation/emission).

Real-time PCR analysis

The NOS-1 cells were seeded and cultured as described above, then after three and seven days in culture, the cells were rinsed twice with PBS (−) and lysed by adding the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was prepared following the manufacturer's instructions. The first strand cDNA was synthesized from the total RNA following the manufacturer's instructions using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). For the PCR reaction, the Brilliant SYBR Green QPCR Master Mix® (Invitrogen) containing the components necessary to carry out PCR amplifications, including Taq DNA polymerase (Nippon gene, Tokyo, Japan) and SYBR Green I® (Stratagene, La Jolla, CA, USA) as the reporter fluorescent dye, ROX as the reference dye and cDNA as the PCR template, were added to a 0.2 μL PCR tube. The primers (for osteocalcin, osteopontin, BMP-2 and integrin β3) that were used in this study are shown in Table 1. The forward and reverse primers were designed on the basis of published nucleotide sequences of the DDBJ. Glyceraldehyde phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control.

The cDNA was amplified under the following conditions with a real-time PCR system (Mx3000P™, Stratagene, La Jolla, CA, USA): 40 cycles at 94ºC for 1 min, 53ºC for 1 min, 72ºC for 1 min. The reactions were performed in triplicate. A melting curve peak was observed for each sample, confirming the purity and specificity of both amplified products. The mRNA level of each gene relative to that of GAPDH was calculated using the "Comparative Quantification" method with the Stratagene® kit.

Table 1 Oligonucleotide primers used for PCR analyses

Gene name	Oligonucleotides (5'→3')	
GAPDH	Forward	GGGAGCCAAAAGGGTCATCATCTC
	Reverse	CCATGCCAGTGAGCTTCCCGTTC
Osteocalcin	Forward	CATGAGAGCCCTCACA
	Reverse	AGAGCGACACCCTAGAC
Osteopontin	Forward	TGAAACGAGTCAGCTGGATG
	Reverse	TGAAATTCATGGCTGTGGAA
BMP-2	Forward	GGGGACTTCTTGAACTTGC
	Reverse	CAGCGTCTCAGTGTCGGGCA
Integrin β3	Forward	GACAAGGGCTCTGGAGACAG
	Reverse	ACTGGTGAGCTTTCGCATCT

Western blot analysis

The NOS-1 cells were again seeded in 60 mm culture dishes at a density of 2×10^5 cells and were cultured with or without FCP. Cells in the FCP group were incubated with an optimal concentration based on the ALP activity assay as described in the aforementioned conditions. After three and seven days of cell culture, the cells were retrieved with a rubber policeman. The cells in each group were lysed in a lysis buffer (1M HEPES, 10% NP-40, 1mM NaF, 1mM Na3VO4, 0.5M EDTA, 100% protease inhibitor cocktail, deionized water) and then they were sonicated with a cell disrupter for 1 min in ice-cold water. After centrifugation of the lysates at 1,000 rpm for 10 min at 4ºC, the supernatants were subjected to a Western blot analysis. The protein concentrations in each group were determined using the micro-Lowry method. A 30 μL sample was denatured in 2×SDS-PAGE sample buffer and separated by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (BIO-RAD, Hercules, CA, USA). The membranes were incubated with 5% (w/v) nonfat milk powder in TBS for 1 h to prevent nonspecific binding. Following the blocking, the membranes were incubated overnight at 4ºC with a primary antibody (Anti-osteopontin: American Research Products Inc, Belmont, MA, USA, Anti-BMP-2 and anti-osteocalcin: Abcam Inc., Cambridge, MA, USA, anti-integrin β3: Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The specific antibody binding was detected with a horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK). The band density of each group was quantified by a densitometric analysis using the Scion Image software program. The ratio of the densitometric value of the FCP group to the value of the control group was then calculated.

Von Kossa staining

The NOS-1 cells were seeded in 35 mm culture dishes at a density of 2×10^5 cells and cultured with α-MEM. After the cells were confluent, they were cultured with 5 mM sodium β-glycerophosphate. An optimal concentration of FCP (based on the ALP activity assay under the aforementioned conditions) was also added in the medium of the FCP group. After seven, 14 and 21 days of culture, the cells were rinsed twice with PBS (−) and fixed for 40 min with 10% formalin, and then were immersed in 5% silver nitrate for 90 min. After they were rinsed thoroughly in distilled water, they were fixed with a 5% sodium thiosulfate solution for 3 min. They were then counterstained with Kernechtrot solution for 3 min and washed thoroughly in distilled water, and finally observed using a phase-contrast microscope. The nodule's area per focal field by the phase-contrast microscopy in each group was analyzed using Scion image software.

Statistical analysis

The statistical significance of differences was determined using a one-way ANOVA and Fisher's PLSD test as a post-hoc analysis for the results of the ALP, cell proliferation study and PicoGreen® DNA assays, Student's *t*-test for the results of the area of cell and nodule studies and densitometric analysis for western blotting using the StatView software program (SAS Institute Inc., Cary, NC, USA). The results were considered to be statistically significant for a p value of <0.05.

RESULTS

ALP activity assay

For all experimental periods, the cell viability was confirmed using a phase contrast microscope. The ALP activity of the control and FCP groups at different concentrations after three days of incubation is shown

in Table 2. The activity of the 0.1% FCP group was significantly higher than that of the other groups ($p<0.05$). Therefore, the 0.1% FCP solution was considered to be the optimal condition for the subsequent studies.

The ALP activities of the control, 0.1% CP and FCP groups after three days of incubation are shown in Fig. 1. The ALP activity of the 0.1% FCP group was significantly higher than that of the other groups ($p<0.01$). After seven days of incubation, the ALP activity of the control and 0.1% FCP groups is 3.06±0.09 and 2.63±0.08 μmol pNPP/mg protein/min, respectively. There was no statistical difference between two groups ($p=0.073$).

Table 2 ALP activity in NOS-1 cells after 3 days of cell culture

Conditions	Activity (means±SE; μmol pNPP/mg protein/min)
Control	2.148±0.062
0.0005% (w/v) fish collagen	2.183±0.082
0.005% (w/v) fish collagen	2.262±0.051
0.05% (w/v) fish collagen	2.155±0.008
0.1% (w/v) fish collagen	3.379±0.037*
0.5% (w/v) fish collagen	2.294±0.039

*: 0.1% (w/v) FCP *versus* other groups ($p<0.05$).
Data are from triplicate samples.

Cell proliferation

To examine the effects of FCP on the proliferation of NOS-1 cells, the total cell number was compared between the control, CP and FCP groups after one, three, five and seven days of incubation. Figure 2 shows the changes in the cell number. There was a significant difference between the FCP and control or CP groups during the experiment ($p<0.05$).

Photographs of the NOS-1 cells in the control and FCP groups after the different lengths of incubation are shown in Fig. 3A. Although the cell clusters in both groups enlarged during the experiment, the cell density in the FCP group was significantly higher than that in the control group on and after three days of incubation (Fig. 3B).

Figure 4 shows the DNA concentrations of the NOS-1 cells in the control, 0.1% CP and FCP groups after the different periods of incubation. Although there were no significant differences between the control and CP groups during the experimental period, the concentration in the FCP group was significantly higher than that of the other groups ($p<0.01$ after five days of incubation, $p<0.05$ after one, three and seven days of incubation).

mRNA expression of osteocalcin, BMP-2, osteopontin and integrin β3

A real-time PCR analysis was performed to investigate the differences in the mRNA expression levels of osteocalcin, BMP-2, osteopontin and integrin β3, which are representative genes related to mineralization. The mRNA expression ratios of the FCP group to the control group after three and seven days of culture are shown in Table 3 (mean±standard error). The results revealed that the expression of all four mRNAs increased after

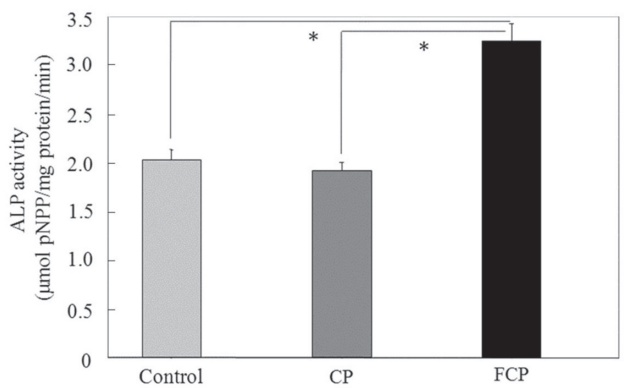

Fig. 1 The ALP activity in NOS-1 cells after 3 days of culture.
*0.1% (w/v) CP *versus* FCP, *versus* control group ($p<0.01$).
The data are from triplicate samples.

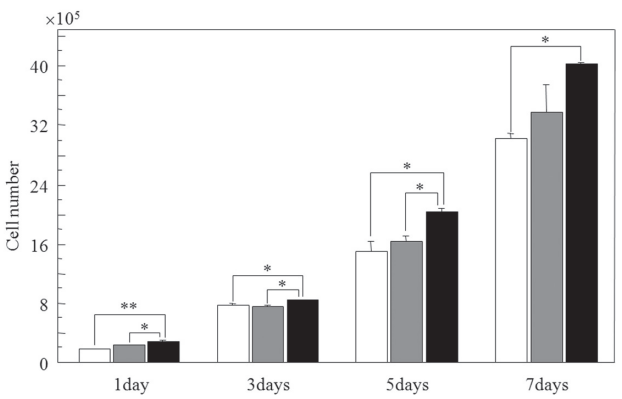

Fig. 2 The numbers of cells after 1, 3, 5 and 7 days of culture with 0.1% (w/v) CP (gray column), or with 0.1% (w/v) FCP (black column) or without 0.1% (w/v) FCP (white column).
*0.1% (w/v) CP *versus* FCP, *versus* the control group ($p<0.05$).
**0.1% (w/v) CP versus FCP, *versus* the control group ($p<0.01$).
The data are from triplicate samples.

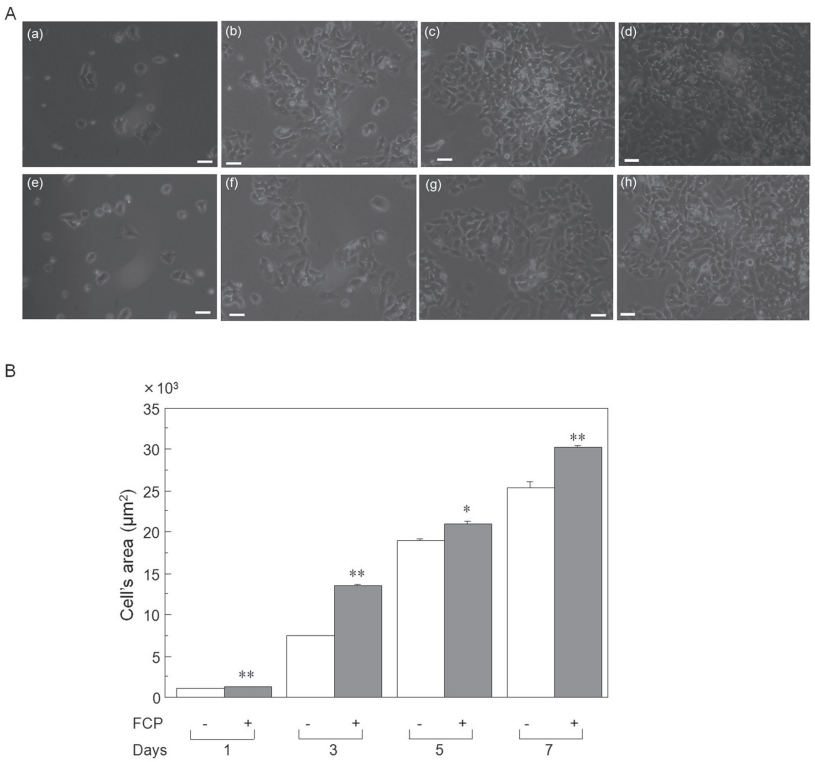

Fig. 3 A: NOS-1 cells cultured for one, three, five, and seven days with (a to d) or without (e to h) 0.1% (w/v) FCP: (a and e) 1 day, (b and f) 3 days, (c and g) 5 days and (d and h) 7 days of cell culture. Scale bar: 15 μm.
B: NOS-1cell's area per focal field by the phase-contrast microscopy at 1, 3, 5, and 7 days of cell culture. The area of cell cluster in each group after 1, 3, 5, and 7 days of culture with (gray column) or without (white column) 0.1% (w/v) FCP are shown graphically.
*0.1% (w/v) FCP *versus* the control group (p<0.05).
**0.1% (w/v) FCP *versus* the control group (p<0.01).
The data are from triplicate samples.

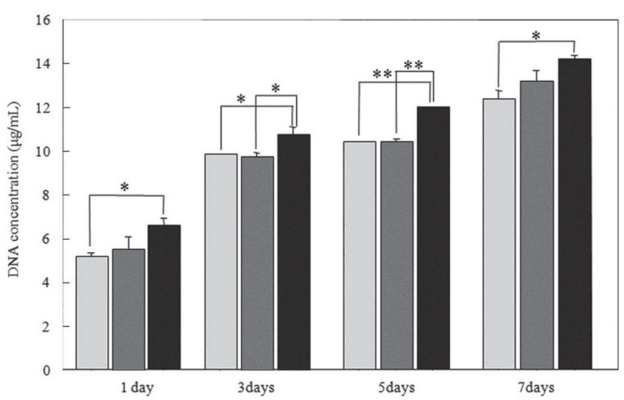

Fig. 4 The DNA concentration of NOS-1 cells cultured for 1, 3, 5 and 7 days with 0.1% (w/v) CP (the middle column) or with (the right column) or without (the left column) 0.1% (w/v) FCP.
*0.1% (w/v) CP *versus* FCP, *versus* the control group (p<0.05).
**0.1% (w/v) CP *versus* FCP, *versus* the control group (p<0.01).
The data are from triplicate samples.

Table 3 Gene expression after 3 and 7 days of culture

Gene name	Ratio (3 days)	Ratio (7 days)
Osteocalcin	3.7±0.1	1.0±0.1
BMP-2	3.4±0.1	1.0±0.1
Osteopontin	1.3±0.1	2.1±0.2
Integrin β3	2.2±0.1	1.9±0.1

Confirmed by semi-quantative RT-PCR. Data are from triplicate samples.

three days of culture supplemented with FCP, whereas the mRNA levels of osteocalcin and BMP-2 decreased to the same level as those in the control group after seven days of culture with FCP. The mRNA expression ratios for osteopontin and integrin β3 were still higher after seven days of culture with FCP.

Evaluation of the protein expression of osteocalcin, BMP-2, osteopontin and integrin β3

The protein levels of these same markers were evaluated by a Western blot analysis. The protein expression ratios of the FCP group to the control group after three and seven days of culture are shown in Fig. 5. These results revealed that the production of the BMP-2 and integrin β3 proteins increased after three days in culture supplemented with FCP ($p<0.01$ for BMP-2, $p<0.05$ for integrin β3), whereas the production of osteocalcin and osteopontin was not significantly different from those of the control group. The production of BMP-2 protein decreased in comparison to that of the control group after seven days of culture with FCP ($p<0.01$). The osteocalcin, osteopontin and integrin β3 protein expression levels showed a trend to increase after seven days of culture with FCP ($p<0.01$ for osteocalcin and osteopontin, $p<0.05$ for integrin β3).

Mineralization study

NOS-1 cells that were incubated for seven, 14 or 21 days are shown in Fig. 6A. Mineralized nodules were presented as dark brown following von Kossa staining. Although the density of mineralized nodules in both groups increased during the experiment, one nodule in the FCP group was significantly larger than that in the control group on and after seven days of incubation (Fig. 6B).

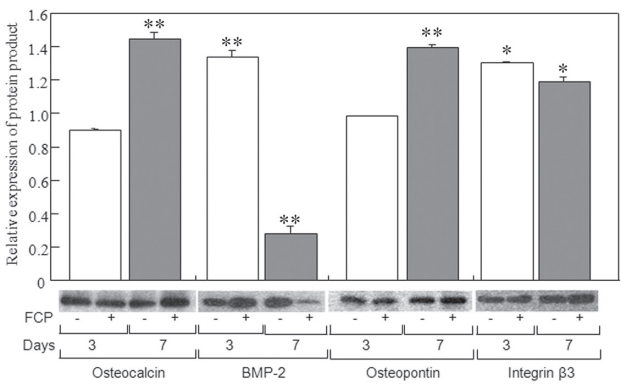

Fig. 5 The results of the Western blot analysis of the expression of osteocalcin, BMP-2, osteopontin and integrin β3 in NOS-1 cells.
The relative expression levels of each protein product after 3 (white column) and 7 (gray column) days of culture with or without 0.1% (w/v) FCP are shown graphically. They were confirmed by the densitometric values. The expression levels were normalized to the densitometric value of the control group.
*0.1% (w/v) FCP *versus* the control group ($p<0.05$).
**0.1% (w/v) FCP *versus* the control group ($p<0.01$).
The data are from duplicate samples.

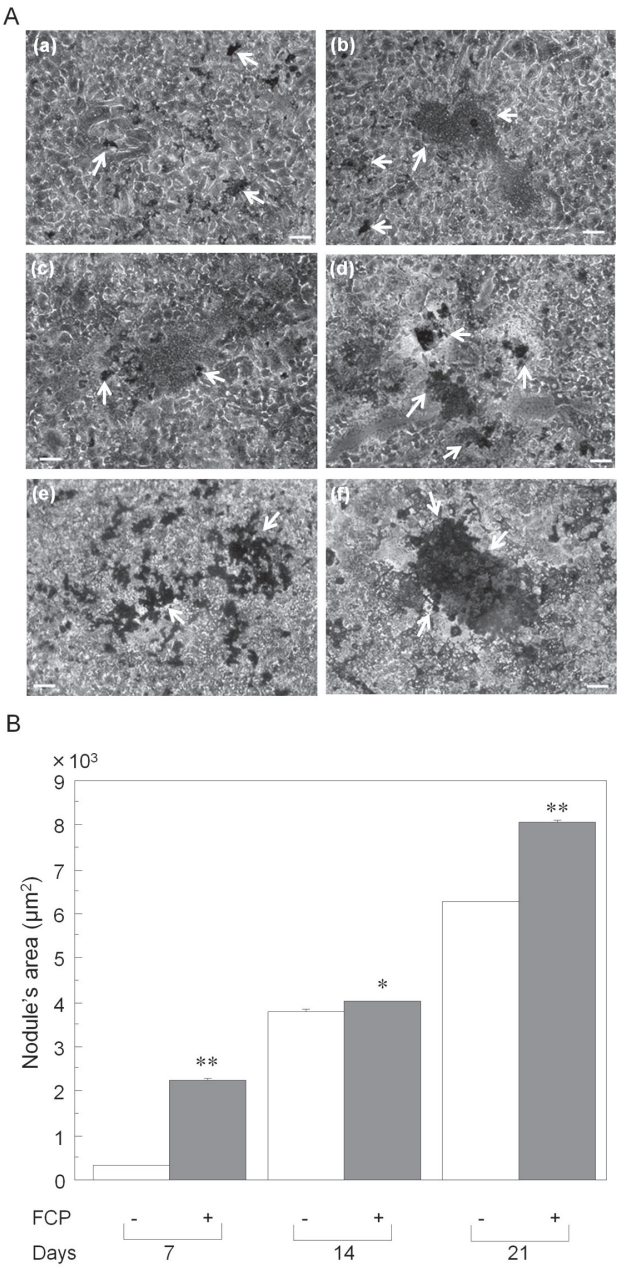

Fig. 6 A: NOS-1 cells cultured for 7, 14 and 21 days with (b, d and f) or without (a, c and e) 0.1% (w/v) FCP after reaching confluence with only α-MEM: (a and b) 7 days, (c and d) 14 days and (e and f) 21 days of cell culture. The arrow shows a mineralized nodule. Scale bar: 15 μm.
B: Nodule's area per focal field by the phase-contrast microscopy at 7, 14, and 21 days of cell culture. The area of nodule in each group after 7, 14, and 21 days of culture with (gray column) or without (white column) 0.1% (w/v) FCP are shown graphically.
*0.1% (w/v) FCP *versus* the control group ($p<0.05$).
**0.1% (w/v) FCP *versus* the control group ($p<0.01$).
The data are from triplicate samples.

DISCUSSION

The concentration of FCP for cell growth, gene and protein expressions related to cell differentiation and mineralization assays was determined primarily based on the highest increased activity (0.1% FCP group) of ALP known as an osteoblastic marker.

A result of cell proliferation study correlated with one of the DNA assay and revealed that the fish collagen stimulated the cell growth. This is consistent with the study reported previously[8], suggesting that the FCP has a biocompatibility.

Although the data by HPLC are different from the well-known amino acid composition of fish collagen, the cell proliferation and gene and protein expression changes related to mineralization increased significantly compared with both the control and casein peptide groups in the present study. These findings suggest that the present FCP has a bioactive effect for osteoblasts. However, further studies are necessary to clarify the exact nature of this effect by specific amino acids and specific molecular weight fractions in the protein. Type I collagen has been reported to facilitate the differentiation of clonal porcine dental follicle cells and bone marrow cells[9-11]. However, the collagen used in those studies was mammalian. Hydrolyzed collagens (2 or 5 kDa) from bovine, porcine and fish origin in the tissue culture medium did not show any significant effects on cell growth after four weeks. However, there was a significant and dose-dependent increase in ALP activity in culture with 2 kDa collagen[12]. The present study indicates that FCP (which is mainly 3 kDa protein) has subcellular effects on the proliferation and differentiation of osteoblasts. These effects probably depend on the origin and molecular weight (MW) of the collagen. The MW of the present FCP was a relatively wide range (500–10,000 Da). These, small peptides could easily come in contact with the osteoblastic cell membrane and accelerate the cellular activities.

ALP is an enzyme that hydrolyses phosphate ester compounds under alkaline conditions and is an early marker of osteoblast differentiation. ALP is generally used as an indicator of early stage of osteoblastic differentiation because a plenty of protein synthesis at seven days of incubation is predominant. The ALP activity is high on the cell membranes of osteoblasts and matrix vesicles at the early stage of culture[13,14]. Therefore, the ALP activity was used in the present study as an indicator to determine the optimal concentration of FCP. A low, 0.1% (w/v), concentration of FCP was the optimal concentration in this study, which is consistent with the study published by Guillerminet *et al.*[12].

Osteocalcin is a mineralization-specific marker because its expression increases as the mineralization increases. The expression of the gene encoding osteocalcin is upregulated by type I collagen in cultured dental pulp cells that have the capacity for mineralization[15]. Although its expression was unchanged after three days of culture in this study, it increased markedly after seven days of culture. A discrepancy between each expressions of mRNA and protein at three and seven days of culture may be due to the differentiation of a translational period in osteocalcin[16]. Osteocalcin is highly expressed during the mineralization stage of bone development. The early expression of osteocalcin in this study demonstrates that FCP could promote the osteoblastic differentiation, even at the early stage.

Osteopontin does not always play an important role in the initial mineralization, because it accumulates whether mineral formation occurs or not[17,18]. Therefore, osteopontin might not be a mineralization-specific factor. Xiao *et al.* reported that nodules of calcification produced from osteoblasts are detected in the matrix, and that there is a correlation between calcification and the distribution of bone sialoprotein and osteopontin[19]. In addition, the osteopontin secreted from the basolateral surfaces of osteoclasts might act as an autocrine factor by binding to the αVβ3-integrin[20]. In the present study, the expression of the osteopontin gene and protein were a little low in comparison to the other three genes after three days in culture, while the expression of osteopontin mRNA increased after seven days of culture. Although further investigation is needed to clarify the molecular events regulating the osteopontin gene, this expression pattern might be characteristic of the application of FCP.

BMP is an osteoblast-specific marker. The autocrine action of BMP, as well as integrin-mediated cell-collagen interactions, are required for osteoblast differentiation, and the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways require MAP kinase activity[21]. Furthermore, the W20-17 cell line (an osteoprogenitor cell lines) produced detectable increases in ALP activity after exposure to rhBMP-2 levels of 5 ng/mL[22]. The ALP activity and BMP-2 expression increased after three days of cell culture in this study. In fact, the expression of both the BMP-2 mRNA and protein were enhanced greatly after three days of culture in this study, which implies the induction of osteoblast differentiation. However, the decrease after seven days of culture indicates that a negative feedback mechanism might act against its overexpression after three days of culture[23].

Integrins are heterodimers consisting of subunits comprising α and β chains, and exist in the cell membrane. Endothelial cells and osteoblasts are generally used to study these proteins, because they are associated with hemostasis, thrombosis[24] and osteoporosis[25]. Furthermore, integrins are essential for cell adhesion, and serve as a mediator between the extracellular matrix and the phosphorylation of downstream signals in the MAPK cascade and other pathways. In addition, the focal adhesion kinase and extracellular signal-related kinase/MAPK are closely associated with the induction of ALP activity in osteoblasts[26]. In this study, the mRNA and protein expression of integrin β3 were increased after both three and seven days of culture. Therefore, FCP might stimulate the osteoblastic function of cells through a complex network of the MAPK cascade and Smad pathway[27].

CONCLUSIONS

The present findings suggest that a low concentration of FCP may possibly improve the proliferation and differentiation of osteoblasts through the expression of ALP, osteocalcin, osteopontin, BMP-2 and integrin β3 at the early stage in cell culture. Furthermore, the present study indicates the potential utility of FCP as a biomaterial for hard tissue repair.

ACKNOWLEDGMENTS

The amino acids present in the FCP were analyzed by the Collagen Biochemistry Laboratory (Director, Prof. Mitsuo Yamauchi), NC Oral Health Institute, USA.

REFERENCES

1) Moskowitz RW. Role of collagen hydrolysate in bone and joint disease. Semin Arthritis Rheum 2000; 30: 87-99.
2) Deal CL, Moskowitz RW. Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. Rheum Dis Clin North Am 1999; 25: 379-395.
3) Koyama Y, Hirota A, Mori H, Takahara H, Kuwaba K, Kusubata M, Matsubara Y, Kasugai S, Itoh M, Irie S. Ingestion of gelatin has differential effect on bone mineral density and body weight in protein undernutrition. J Nutr Sci Vitaminol 2001; 47: 84-86.
4) Ueno A, Yamashita K, Miyoshi K, Horiguchi T, Ruspita I, Abe K, Noma T. Soluble matrix from osteoblastic cells induces mineralization by dental pulp cells. J Med Invest 2006; 53: 297-302.
5) Eastoe JE. The amino acid composition of fish collagen and gelatin. Biochem J 1957; 65: 363-368.
6) Giraud-Guille MM, Besseau L, Chopin C, Durand P, Herbage D. Structural aspects of fish skin collagen which forms ordered arrays *via* liquid crystalline states. Biomaterials 2000; 21: 899-906.
7) Peterson GL. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal Biochem 1977; 83: 346-356.
8) Song E, Kim SY, Chun T, Byun HJ, Lee YM. Collagen scaffolds derived from a marine source and their biocompatibility. Biomaterials 2006; 27: 2951-2961.
9) Tsuchiya S, Honda MJ, Shinohara Y, Saito M, Ueda M. Collagen type I matrix affects molecular and cellular behavior of purified porcine dental follicle cells. Cell Tissue Res 2008; 331: 447-459.
10) Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction. J Cell Physiol 2000; 184: 207-213.
11) Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J Biochem 2001; 129: 133-138.
12) Guillerminet F, Beaupied H, Fabien-Soule V, Tomé D, Benhamou CL, Roux C, Blais A. Hydroluzed collagen inproves bone metabolism and biochemical parameters in ovariectomized mice: An *in vitro* and *in vivo* study. Bone 2010; 46: 827-834.
13) Yamada S, Ohara N, Hayashi Y. Mineralization of matrix vesicles isolated from a human osteosarcoma cell line in culture with water-soluble chitosan-containing medium. J Biomed Mater Res A 2003; 66: 500-506.
14) Yamada S, Ganno T, Ohara N, Hayashi Y. Chitosan monomer accelerates alkaline phosphatase activity on human osteoblastic cells under hypofunctional conditions. J Biomed Mater Res A 2007; 83: 290-295.
15) Nagata T, Bellows CG, Kasugai S, Butler WT, Sodek J. Biosynthesis of bone proteins [SPP-1 (secreted phosphoprotein-1, osteopontin), BSP (bone sialoprotein) and SPARC (osteonectin)] in association with mineralized-tissue formation by fetal-rat calvarial cells in culture. Biochem J 1991; 274: 513-520.
16) Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. Global quantification of mammalian gene expression control. Nature 2011; 473: 337-342.
17) Kasugai S, Nagata T, Sodek J. Temporal studies on the tissue compartmentalization of bone sialoprotein (BSP), osteopontin (OPN), and SPARC protein during bone formation *in vitro*. J Cell Physiol 1992; 152: 457-477.
18) Mizuno M, Miyamoto T, Wada K, Watatani S, Zhang GX. Type I collagen regulated dentin matrix protein-1 (Dmp-1) and osteocalcin (OCN) gene expression of rat dental pulp cells. J Cell Biochem 2003; 88: 1112-1119.
19) Xiao Y, Haase H, Young WG, Bartold PM. Development and transplantation of a mineralized matrix formed by osteoblasts *in vitro* for bone regeneration. Cell Transplant 2004; 13: 15-25.
20) Chellaiah MA, Hruska KA. The integrin αvβ3 and CD44 regulate the actions of osteopontin on osteoclast motility. Calcif Tissue Int 2003; 72: 197-205.
21) Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, Extracellular matrix, and Mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MT3T3-E1 cells. J Bone Miner Res 2002; 17: 101-110.
22) Blum JS, Li RH, Mikos AG, Barry MA. An optimized method for the chemiluminescent detection of alkaline phosphatase levels during osteodifferentiation by bone morphogenetic protein 2. J Cell Biochem 2001; 80: 532-537.
23) Martinovic S, Borovecki F, Miljavac V, Kisic V, Maticic D, Francetic I, Vukicevic S. Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation. Arch Histol Cytol 2006; 69: 23-36.
24) Petrich BG, Marchese P, Ruggeri ZM, Spiess S, Weichert RA, Ye F, Tiedt R, Skoda RC, Monkley SJ, Critchley DR, Ginsberg MH. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. J Exp Med 2007; 204: 3103-3111.
25) Zhao H, Kitaura H, Sands MS, Ross FP, Teitelbaum SL, Novack DV. Critical role of β3 integrin in experimental postmenopausal osteoporosis. J Bone Miner Res 2005; 20: 2116-2123.
26) Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-β receptor down-regulation by collagen-α2β1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem 1997; 272: 29309-29316.
27) Chen G, Deng C, Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci 2012; 8: 272-288.