

The effect of different molecular weight collagen peptides on MC3T3-E1 cells differentiation

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Abstract. Our study aimed at investigating the effect of different molecular weight bovine collagen peptides, namely CH878, CH1370, CH2900, and CH7747 on the differentiation of MC3T3-E1 cells. Osteogenic differentiation of MC3T3-E1 cells was assessed by a series of specific assays, after culturing of cells in the presence of collagen peptides. Alkaline phosphatase activity (ALP) was evaluated by NBT/BCIP staining. Osteocalcin expression was determined by a radioimmunity method. Mineralization was quantified by Alizarin Red staining. ALP staining results demonstrated that the ALP staining of cells after culture in the presence of collagen peptides were significantly higher than the control group ($P < 0.05$), indicating the promotion of ALP activity in MC3T3-E1 cells by these peptides. Radioimmunity results demonstrated that collagen peptides groups were all significantly higher than the control group ($P < 0.01$). Alizarin Red staining results demonstrated that CH1370, CH2900, and CH7747 significantly promoted the formation of mineralized bone matrix. We therefore conclude that CH1370, CH2900, and CH7747 play an active role in the differentiation of MC3T3-E1 cells. Based on the above results, we provide molecular basis for further development of collagens with different molecular weight for the prevention and treatment of osteoporosis.

Key words: Different molecular weight collagen peptides, MC3T3-E1 cells, differentiation

1. Introduction

The excessive bone resorption and lack of bone formation can cause reduced bone density and damaged bone microstructure, and finally result in osteoporosis [1]. As the development of society, the trend of world population aging is increasing. The age-related bone loss and senile osteoporosis

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will increase the risk of bone fractures and the outbreaks of bone disease, which will not only cause huge economic losses but also induce chronic pain, disability, and death [2]. Correlations between osteocalcin level and bone turnover rates have been reported as indicator of bone remodelling. The treatment for osteoporosis mainly relies on western medicine; however there are some side effects, for instance alendronate is a diphosphonate and it has the risk of increasing gastrointestinal bleeding [3]. Collagen peptides, the collagen hydrolysates, are produced by hydrolysis of polypeptides from gelatin mixture. Because they can be used as potential oral supplements for bone and joint tissues, and they can also enhance the bone density of ovariectomized rats, collagen peptides draw wide attention of researchers [4]. It is reported that the bioactivities of collagen peptides are not only related to the source of collagen, but also correlated with their molecular weight, such as antioxidant activities found in active peptides derived from fish or squid skin gelatins [5]. However the optimal molecular weight range of collagens for the osteoporosis treatment has not been reported yet. The main purpose of our study is to investigate the effect of different molecular weight collagen peptides on the differentiation of osteoblast *in vitro* and further determine the optimal molecular weight range of collagens that would enhance osteogenesis. We first isolated and cultured rat osteoblast (MC3T3-E1 cells), and characterized the effect of CH878, CH1370, CH2900, and CH7747 bovine collagen peptides on the propensity of specific osteoblast differentiation markers, such as alkaline phosphatase activity, osteocalcin expression and mineralization. The results of this paper provide new clues for the potential use of collagen peptides in the prevention and treatment of osteoporosis.

2. Materials and methods

2.1. Reagents and instruments

Bovine bone gelatin was from Dongbao Biotechnology Co. Ltd., Baotou, China. we have digested bovine bone gelatin to peptides (0~10,000Da) and then separated the peptides with different molecular weight (CH878, CH1370, CH2900, and CH7747 – numbers represent weight in Daltons-, whose contents within $\pm 50\%$ average molecular weight were 81.1%, 88.4%, 89.3, 77.6%) by UF membrane filtration. Mouse pre-osteoblast cell line MC3T3-E1 subclone 4 cells (ATCC, Bethesda, MD) [6] were obtained from the Chinese Medicine Academy of Sciences Cell Bank.

DMEM medium and premium Fetal Bovine Serum (FBS) were from Gibco. Alizarin Red S, dexamethasone, β -glycerol phosphate disodium salt, ascorbic acid phosphate, dimethylsulphoxide (DMSO), and trypsin were from Sigma, USA. ALP test kit was from Pik-day Biotechnology Institute. OC test kit was from Beijing North Institute of Biological Technology. Penicillin and streptomycin were from North China Pharmaceutical Group Corporation. The other commercially available reagents were analytical grade.

HERAcell 150i CO₂ incubator was from Heraeus, Germany; IMT-2 inverted research microscope was from Olympus, Japan; 550 ELISA analyzer was from BIO-RAD, USA. The cell culture dishes and 24-well culture plates were from Costar, USA.

2.2. Methods

2.2.1. The cultivation and differentiation of MC3T3-E1 cells

The cells were cultured routinely in DMEM with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37°C, under 5% CO₂ and saturated humidity. The cells were regularly passaged when

they reached semi-confluence. MC3T3-E1 cells at passage 3 were used for experiments.

To determine the effect of different molecular weight collagen peptides on MC3T3-E1 cells differentiation, we prepared different molecular weight collagen peptides at 0.75, 1.5 and 3 mg/mL concentration dissolved in hG-DMEM with 10% FBS. MC3T3-E1 cells were seeded on 24-well plates at a density of 3×10^3 cell/well with four duplicate wells per treatment, and induced by osteoinductive factors (50 µg/mL ascorbic acid phosphate, 10 mM β-glycerol phosphate disodium salt, 10 nmol/L dexamethasone) next day after seeding, then treated for 14 days with the above collagen peptides or without collagen peptides (CN), and fresh media after treated with collagen peptides or CN and osteoinductive factors were added in cells every 3 days during the entire assay period.

2.2.2. The ALP staining of cells

After 14 days, the cells were rinsed with PBS twice, and fixed with 4% paraformaldehyde for 30 minutes, then they were rinsed with PBS twice and the ALP activities were tested according to the recommended protocol. After the last wash, the wash solution was removed and an appropriate amount of BCIP/NBT staining working solution was added to cover the samples. The plate was incubated in the dark at room temperature for 5 to 30 minutes or longer until the desired color develops. The principle is that BCIP/NBT is the common substrate for ALP; under the catalysis of ALP, BCIP can be hydrolyzed and produce strong reactive product which can react with NBT and form insoluble dark blue to blue-violet product. The plate was rinsed with deionized water twice. The images of stained cells were scanned using a scanner (Canoscan lide 100, Cano, Japan). Quantitative analyses of mean density values of ALP staining were performed using Image Software Pro Plus ipwin32.

2.2.3. The determination of osteocalcin protein expression in cell culture

After 14 days, cell culture media were used for osteocalcin/bone GLA protein (BGP) content assay. The test was following the instructions in the Carboxyglutamic Acid (BGP) Radioimmunoassay Kit. The BGP obtained from standards or samples and added ^{125}I -BGP compete for the same specific antibody; the amount of ^{125}I -BGP that binds to an antibody is a function of the BGP contents in the standards or samples. Using immune-separation reagents (rabbit anti-BGP antibody and donkey anti-rabbit immune separating agent) to separate the bound (B) and free (F) parts, the radioactivity of the bound parts were determined and the corresponding binding rate was calculated. Then, the standard inhibition curve was drawn according to the known BGP contents of the standards and their corresponding binding rates. The standard curve was used to identify, the BGP contents of the samples according to their binding rates. The standard inhibition curve was drawn according to the known BGP contents in the standards and the corresponding binding rates. From the standard curve we can read the BGP contents in the samples according to their binding rates.

2.2.4. Alizarin red staining and mineralization quantification

To measure the calcium nodule formation from the extracellular matrix calcium deposition, we used the Alizarin Red which binds to calcium minerals in the extracellular matrix. Alizarin Red staining indicates the deposition of minerals into the matrix. After 14 days, the media were discarded. Cells were rinsed with PBS twice, and fixed with 4% paraformaldehyde for 30 minutes at 4 °C, and then they were rinsed with deionized water. MC3T3-E1 cells were stained in 40 mmol/L Alizarin red solution (pH=4.4) for 10 minutes and then rinsed twice with deionized water. The images of stained cells were scanned using a scanner (Canoscan lide 100, Cano, Japan). Quantitative analyses of mean density values of ALP staining were performed using Image Software Pro Plus ipwin32.

2.2.5. Statistical analysis

All numerical data are expressed as the mean \pm s.d. All statistical analyses were performed with SPSS for Windows (version 17.0, IBM, Armonk, NY, USA). One-way ANOVA followed by "LSD" or Dunnett's post-hoc test was used to determine statistical differences. $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. ALP content in MC3T3-E1 cells treated with different molecular weight collagen peptides

ALP is an early marker for osteogenesis. After 14 days, with the increase of collagen peptides concentration, the ALP staining becomes deeper and deeper (see Figure 1). The mean density of ALP staining showed no difference between cells treated with 0.75 mg/mL above collagen peptides and CN cells (Table 1). The mean density of ALP staining treated with 1.5 mg/mL CH1370 were increased significantly compared to that of the CN ($P < 0.05$). Obviously, compared to the CN group, there was significant difference of ALP staining after treatment with 3 mg/mL above collagen peptides ($P < 0.05$), indicating that different molecular weight collagen peptides can promote the ALP expression in MC3T3-E1 cells.

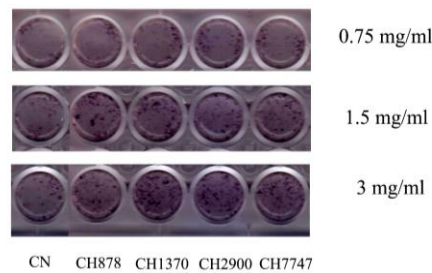


Fig. 1. The ALP content in MC3T3-E1 cells treated with different molecular weight collagen peptides.

Table 1

The mean density values of ALP staining of the different molecular weights of collagen peptides

Mean Density	CN	CH878	CH1370	CH2900	CH7747
0.75 mg/mL	0.37 \pm 0.01	0.37 \pm 0.01	0.38 \pm 0.01	0.38 \pm 0.00	0.38 \pm 0.01
1.5 mg/mL	0.40 \pm 0.00	0.40 \pm 0.01	0.41 \pm 0.01*	0.41 \pm 0.00	0.41 \pm 0.01
3 mg/mL	0.41 \pm 0.00	0.42 \pm 0.01*	0.44 \pm 0.01**	0.43 \pm 0.01*	0.42 \pm 0.01*

Note: Data are expressed as means \pm SD ($n = 4$). * $P < 0.05$ vs. CN. ** $P < 0.01$ vs. CN.

Table 2

The OC content in MC3T3-E1 cells treated with different molecular weight collagen peptides

ng/ml	CN	CH878	CH1370	CH2900	CH7747
0.75 mg/mL	0.27 \pm 0.03**	1.82 \pm 0.13*	0.40 \pm 0.08**	0.31 \pm 0.09	0.26 \pm 0.07
1.5 mg/mL	0.29 \pm 0.01	2.34 \pm 0.16**	0.55 \pm 0.05**	0.41 \pm 0.04**	0.42 \pm 0.04**
3 mg/mL	0.36 \pm 0.02	2.83 \pm 0.19**	0.88 \pm 0.04**	0.74 \pm 0.09**	0.79 \pm 0.06**

Note: Data are expressed as means \pm SD ($n = 4$). * $P < 0.05$ vs. CN. ** $P < 0.01$ vs. CN.

3.2. The OC content in MC3T3-E1 cells treated with different molecular weight collagen peptides

Osteocalcin is a specific non-collagenous bone matrix protein synthesized and secreted by bone cells. The OC contents measured by radioimmunoassay at the 15th day in MC3T3-E1 cells treated with CH878, CH1370, CH2900, and CH7747 were higher than CN group (see Table 2), and the differences are significant ($P < 0.01$), indicating that different molecular weight collagen peptides can promote the OC content in late stage MC3T3-E1 cells.

3.3. The mineralization in MC3T3-E1 cells treated with different molecular weight collagen peptides

The mineralization is critical for bone formation. After 14 days, the Alizarin Red staining results were shown in Figure 2. The MC3T3-E1 cells treated with 0.75, 1.5 and 3 mg/mL CH1370, CH2900, and CH7747 had darker colors compared to the CN group, while the MC3T3-E1 cells treated with 0.75, 1.5 and 3 mg/mL CH878 had a lighter color compared to the CN group (see Figure 2). The above CH1370, CH2900, and CH7747 groups showed significant higher the mean density of alizarin red staining than the CN group ($P < 0.01$, Table 3); while the CH878 group showed a significant lower the mean density of alizarin red staining than the CN group ($P < 0.01$), further indicating that CH1370, CH2900, and CH7747 can promote the mineralization in MC3T3-E1 cells.

4. Discussion

Collagen peptides have been reported to have numerous distinct bioactivities, including anti-oxidization, anti-aging, promoting the repair and growth of corneal epithelial cells, promoting the metabolism of skin collagen, immunomodulation, et al. [7, 8]. The bioactivity of collagen is directly correlated to its molecular weight. Han and colleagues found that the Halibut skin collagen peptides (600-1,800Da) could inhibit angiotensin I-converting enzyme activity in a molecular weight-dependent manner; low molecular weight collagens are more potent inhibitors for angiotensin I-converting enzyme [9]. Lin, et al. have obtained collagen peptides with molecular weight of less

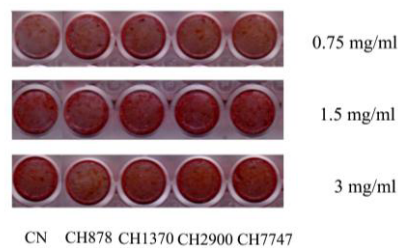


Fig. 2. The mineralization effects of the MC3T3-E1 cells treated with different molecular weights of collagen peptides.

Table 3

The mean density values of alizarin red staining of the different molecular weights of collagen peptides

Mean Density	CN	CH878	CH1370	CH2900	CH7747
0.75 mg/mL	0.47±0.00	0.50±0.00**	0.52±0.01**	0.51±0.01**	0.50±0.01**
1.5 mg/mL	0.49±0.01	0.46±0.02	0.50±0.00	0.51±0.01*	0.51±0.01**
3 mg/mL	0.53±0.01	0.49±0.00**	0.53±0.01	0.55±0.01**	0.55±0.00**

Note: Data are expressed as means ± SD ($n = 4$). * $P < 0.05$ vs. CN. ** $P < 0.01$ vs. CN.

than 10,000Da by pepsin digestion of Squid skin gelatin and showed that the collagens smaller than 2,000Da have better anti-ACE activity [10]. With pronase E digestion of Alaska cod skin gelatin, Kim SK and colleagues produced collagens with molecular weight of 1,500-4,500Da, which have excellent anti-oxidation activity and can be used as a natural anti-oxidant [11]. In 2005, Kim SK separated the trypsin digestion products of cod gelatin with chromatographic method and identified the peptide with highest anti-oxidation activity, the sequence of which is His-Gly-Pro-Leu-Gly-Pro-Leu (797Da) [12]. Zhang applied alkaline protease to digest tilapia skin and produced peptides with different molecular weights, 48.28% with 926Da, 38.89% with 675Da, 12.83 with 350Da, all of which have strong antioxidant and hydroxyl radical scavenging activities [13].

The hydrolyzed collagens have been recognized as safe food ingredients [14, 15]. Clinical studies revealed that the hydrolyzed collagens can not only improve joint function [16] and reduce joint pain [17], but also relieve symptoms of osteoporosis. By feeding ovariectomized rats with oral collagen peptides, Nomura, et al. reported that collagens can increase rat bone density and improve the spine function. Collagens are peptide mixtures with different molecular weights. Hydrolyzed pigskin collagens smaller than 3,000Da are reported to prevent the bone loss due to ovariectomization, and improve microstructure of lumbar bones [18]. Oral medications of collagens smaller than 5,000Da can significantly increase the bone density and strength of ovariectomized rats. However, oral medication of casein did not show such effect, suggesting the possible correlation between molecular weight of collagens and its bone activity.

Although a large number of clinical studies have demonstrated the effect of collagens in osteoporosis and arthritis patients, the optimal molecular weight range of collagens for the osteoporosis treatment and the bioactivities is not clear. In present study, we digested bovine bone gelatin to peptides (0~10,000Da) and then separated the collagens peptides with different molecular weight by UF membrane filtration. Osteogenic differentiation of MC3T3-E1 cells was assessed by a series of specific assays. Our research provides valuable new thoughts for the manufacturing of bioactive collagens.

ALP is a molecular marker for bone formation and osteoblast differentiation. ALP from osteoblast cells are secreted to extracellular matrix together with Ca^{2+} salt, which increase the local phosphate concentration and promote the mineralization of matrix. In the late stage of osteoblast differentiation, cellular ALP level is decreased when the cells begin mineralization [19]. We have showed that CH1370 and CH7747 can promote ALP expression in MC3T3-E1 cells, while CH878 has the opposite effect. The results indicate that the ALP effect is related to the molecular weight distribution of collagens.

Osteocalcin is the most abundant protein in bone matrix and is critical for the bone mineralization. Osteocalcin level can be used as a specific, sensitive biochemical marker for the osteoblast cell, especially for those newly differentiated [20]. The formation of osteoblast mineralization nodules reflects the ability of bone cell differentiation. Results here showed that different molecular weight collagen peptides can increase the OC level in MC3T3-E1 cells and mineralization of osteoblasts, similar as our previous results [21].

The results here provide molecular basis for the development of collagens with different molecular weight for the prevention and treatment of osteoporosis and arthritis.

Acknowledgments

This work was supported by The Cooperation Project from Technical Institute of Physics and

Chemistry, CAS (Grant No. LHS- DBSW2013-01). The funding body had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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