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Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage *in vitro* and *in vivo*

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Summary

Objective: To investigate the direct effect of prolyl-hydroxyproline (Pro-Hyp) on chondrocytes under *in vivo* and *in vitro* conditions in an attempt to identify Pro-Hyp as the bioactive peptide in collagen hydrolysate (CH).

Methods: The *in vivo* effects of CH and Pro-Hyp intake on articular cartilage were studied by microscopic examination of sections of dissected articular cartilage from treated C57BL/6J mice. In this study, mice that were fed diets containing excess phosphorus were used as an *in vivo* model. This mouse line showed loss of chondrocytes and reduced thickness of articular cartilage, with abnormality of the subchondral bone. The *in vitro* effects of CH, Pro-Hyp, amino acids and other peptides on proliferation, differentiation, glycosaminoglycan content and mineralization of chondrocytes were determined by MTT activity and staining with alkaline phosphatase, alcian blue and alizarin red. Expression of chondrogenesis-specific genes in ATDC5 cells was determined by semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR).

Results: *In vivo*, CH and Pro-Hyp inhibited the loss of chondrocytes and thinning of the articular cartilage layer caused by phosphorus-induced degradation. In the *in vitro* study, CH and Pro-Hyp did not affect chondrocyte proliferation but inhibited their differentiation into mineralized chondrocytes. A combination of amino acids such as proline, hydroxyproline and prolyl-hydroxyprolyl–glycine did not affect chondrocyte proliferation or differentiation. Moreover, CH and Pro-Hyp caused two and threefold increases, respectively, in the staining area of glycosaminoglycan in the extracellular matrix of ATDC5 cells. RT-PCR indicated that Pro-Hyp increased the aggrecan mRNA level approximately twofold and decreased the *Runx1* and osteocalcin mRNA levels by two-thirds and one-tenth, respectively.

Conclusion: Pro-Hyp is the first bioactive edible peptide derived from CH to be shown to affect chondrocyte differentiation under pathological conditions.

Key words: Collagen hydrolysate, Prolyl-hydroxyproline, Chondrocyte, Articular cartilage degradation, Extracellular matrix.

Introduction

Collagen hydrolysate (CH), a heterogeneous mixture of oligopeptides and polypeptides similar to gelatin, has been used therapeutically as a dietary supplement to improve conditions of joints. Some animal experiments have suggested that oral ingestion of CH might have beneficial effects on joint conditions such as osteoarthritis (OA)[1,2]. CH is absorbed in its high-molecular-weight form, containing peptides of 2.5–15 kDa[3]. A recent study reported detection of peptides such as prolyl proline (Pro-Pro), alanyl hydroxypropyl glycine (Ala-Hyp-Gly), prolyl-hydroxyproline (Pro-Hyp), prolyl hydroxypropyl glycine (Pro-Hyp-Gly), isoleucyl hydroxyproline (Ile-Hyp), leucyl hydroxyproline (Leu-Hyp) and phenylalanyl hydroxyproline (Phe-Hyp) in human venous blood after ingestion of CH. Pro-Hyp was the most prevalent among those peptides[4,5].

Many reports indicate that various peptides obtained from CH show biological activity. For example, the Asp–Gly–Glu–Ala tetrapeptide regulated the expression of osteoblast-related genes in the bone marrow[6]. Furthermore, the Pro-Hyp dipeptide was suggested to be involved in platelet aggregation[7]. Correspondingly, a hydrogel containing peptides from collagen has been used as a scaffold for a true cartilage-like extracellular matrix in regenerative medicine for effective and lasting repair of articular cartilage[8]. These findings suggest that some peptides that are metabolites act as bioactive peptides and functional molecules in some tissues, and that CH contains bioactive peptides that affect cartilage homeostasis.

This study aimed to examine the protective effect of CH and its specific dipeptide, Pro-Hyp, in relation to primary degenerative articular cartilage of mice.

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Materials and methods

CH, AMINO ACIDS, AND PEPTIDES

Enzymatic CH of porcine skin gelatin (01-JP) was a kind gift from Nitta Gelatin (Osaka, Japan). This hydrolysate was of food grade and is commercially available. CH was a mixture of peptides of various molecular weights (average molecular weight of 5000 Da), including dipeptides and tripeptides. Gly, Pro and Hyp were purchased from Sigma Aldrich (Tokyo, Japan). The peptides Pro-Hyp and Gly–Pro-Hyp were purchased from PH Japan (Hiroshima, Japan) and used at 95% purity.

ANIMALS

Ten-week-old male C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). All the diets used in *in vivo* experiments were modifications of the AIN-93G composition, and we referred to the methods of Morishita *et al.*[9]. The diet compositions are shown in Table I. Potassium dihydrogen

Table I
Compositions of experimental diets

g/kg Diet	High Pi diet			
	N	C	CH	Pro-Hyp
Casein*	200	150	150	150
Lard*	58.3	58.3	58.3	58.3
Corn oil*	11.7	11.7	11.7	11.7
Mineral mixture*	35	35	35	35
Vitamin mixture*	10	10	10	10
Sucrose†	100	100	100	100
Cornstarch*	472.95	472.95	472.95	472.95
Cellulose*	50	50	50	50
KH_2PO_4†	–	59.05	59.05	59.05
L-cystine*	3	3	3	3
Gluten†	–	50	–	47
CH‡	–	–	50	–
Pro-Hyp§	–	–	–	3

*Oriental yeast (Chiba, Japan), AIN-93G was used as a vitamin and mineral mixture composition.

†WAKO (Tokyo, Japan).

‡Nitta gelatin (Osaka, Japan).

§PH JAPAN (Hiroshima, Japan).

phosphate anhydrous (KH_2PO_4) was used as the Pi source and added instead of cornstarch. Gluten, CH or Pro-Hyp was added instead of casein. Gluten hydrolysate from corn (Sigma Aldrich) was used as the negative control. CH contained approximately 6% Pro-Hyp. The mice were randomly assigned to four groups of six mice each: a normal (N) group fed a standard diet containing 0.2 g Pi/100 g; a control (gluten) group fed a diet containing 1.2 g Pi and 5 g gluten/100 g; a CH group fed a diet containing 1.2 g Pi and 5 g porcine skin gelatin/100 g; and a Pro-Hyp group fed a diet containing 1.2 g Pi and 0.3 g Pro-Hyp/100 g. The mice were allowed free access to food and water during the 3-week feeding period. Blood samples were drawn by cardiac puncture of anesthetized mice. The blood samples were stood for 1 h at room temperature and centrifuged at 2000 g for 15 min. The resulting supernatants were employed as serum samples, which were stored at −20°C until use. Serum inorganic P and Ca were measured with Phospher C test WAKO and Calcium E test WAKO, respectively (Wako Pure Chemical, Osaka, Japan). The animals were housed under conditions of constant temperature (20–22°C) and humidity (45–50%), and a standard 12-h light/dark cycle. The study was performed in accordance with the National Institutes of Health (NIH) institutional guidelines for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee of The University of Josai, Saitama, Japan.

HISTOLOGICAL COMPARISON

Dissected articular cartilage was immediately immersion-fixed overnight in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, at 4°C. Articular cartilage sections were cut at a thickness of 10 μm at −22°C with a cryomicrotome (Leica, CM3050S) and stained with Mayer's hematoxylin (H). Each section was compared at the same thickness by measuring instrument of cryomicrotome. Articular cartilages of the same thickness were compared. Each section was observed at ×400 or ×200 magnification, and the shape of chondrocytes, distribution of chondrocytes, thickness of articular cartilage and morphology of subchondral bone were compared. Apoptosis was assessed using an *In Situ* Cell Death Detection Kit (Roche Applied Science) and by observation using the 4′,6-diamino-2-phenylindole (DAPI) fluorescence method.

MICRO-COMPUTED TOMOGRAPHY (CT)

All measurements were performed with a micro-CT desktop system, which is based on a combination of X-ray projection microscopy and a tomographic reconstruction technique (Skyscan 1172, Belgium). In this system, an air-cooled point X-ray source (focal spot size ∼8 μm in diameter, maximum voltage 80 kV) was used to illuminate the object with a divergent beam. All samples and phantoms were scanned under identical conditions. Scanning parameters were as follows: anode voltage, 80 kV; rotation step, 0.5°; and exposure time, 7 s per view. After scanning, virtual cross-sections through the bone were reconstructed with a 32-bit dynamic range and converted into 8-bit map images according to the selected density window using CT-analyzer software.

CELLS AND CULTURE CONDITIONS

ATDC5 cells, a murine chondrocytic cell line, were prepared as described previously[10]. Briefly, the basic medium consisted of dulbecco's modified eagle medium (DMEM)/F12 medium (Invitrogen) containing 1 mM phosphorus, 0.25 mM glycine and 0.15 mM proline. Hydroxyproline was not included in the basic medium. Peptone, a pancreatic digest of casein purchased from Becton–Dickinson, was used as a negative control of CH in the *in vitro* study. In the experiments, the ATDC5 cells were cultured in four different media: the basic medium, and the basic medium supplemented with 1 mg/ml peptone (Pe), 1 mg/ml CH or 2.5 mM Pro-Hyp. Methyl-thiazol-tetrazolium (MTT) assay was performed after 1 day of culture, alkaline phosphatase (ALP) staining was performed after 5 days of culture, and alcian blue (AB) staining and alizarin red (AR) staining were performed after 35 days of culture. In addition, amino acids such as proline, hydroxyproline, and/or glycine, dipeptides or tripeptides were added to the basic culture medium to a final concentration of 2.5 mM before the MTT assay on day 1 of culture and ALP staining on day 5 of culture.

RNA EXTRACTION AND RT-PCR

ATDC5 cells were cultured in 6-cm dishes by seeding at approximately 5×10^5 cells per dish. After culturing for 1 day, the medium was replaced by one of four media: basic medium, 1.25 mM proline and 1.25 mM hydroxyproline (Pro and Hyp), 2.5 mM Pro-Hyp or 2.5 mM Pro-Hyp–Gly. The cells in each of these media were cultured for 3, 24 or 72 h, and then the total RNA was extracted with TRIzol for use in RT-PCR. The method for RNA extraction and the conditions for RT-PCR for type II collagen (Col2), osteocalcin (OCL), *Smad2*, *Smad4* and *Runx2* were as described previously[10,11]. The primer sequences are shown in Table II.

STATISTICAL ANALYSIS

The results are expressed as the mean ± standard deviation (SD). *In vivo* experiments were carried out $n = 6$ animals each groups. Welch's *t* test was used to identify the 'number of cells' and 'thickness of cartilage' differences between control group and Pro-Hyp group. *In vitro* experiments were carried out using five wells for each medium. Each experiment was repeated three times, and representative results are shown. Multiple comparison of mean values was performed by Dunnett's *t* test. The *P* value refers to comparison of a measured parameter in a test medium with that in the basic medium. Statistical significance was set at alpha = 0.05 for one-side test. All statistical computations were performed using SAS version 9.1.3 (SAS Institution, Cary, NC).

Table II
Primers and PCR conditions

Primer	Sequence	Annealing (°C)	Cycles
Runx1 (upstream)	5′-acttcctctgctccgtgcta-3′	58	28
Runx1 (downstream)	5′-ggtagcgagattcaacgacc-3′		
Aggrecan (upstream)	5′-cacgctacaccctggactttg-3′	58	28
Aggrecan (downstream)	5′-ccatctcctcagcgaagcagt-3′		
PEPT1 (upstream)	5′-gcgaggtggtcttctctgtc-3′	58	30
PEPT1 (downstream)	5′-cagaagcaatgaggcaaaca-3′		
PEPT2 (upstream)	5′-tgtcctggttacagcagcag-3′	58	30
PEPT2 (downstream)	5′-caaaacgaattcagcccact-3′		

Results

ADMINISTRATION OF CH AND PRO-HYP AMELIORATES HISTOLOGICAL CHANGES IN PHOSPHORUS-INDUCED CARTILAGE DEGRADATION IN MICE

We examined whether CH and Pro-Hyp affected the articular cartilage of mice *in vivo*. In this study, mice that were fed diets containing excess phosphorus (C) were used as controls (Fig. 1). The final weights and bone lengths of mice were not significantly different among the four groups (data not shown), and neither were the serum inorganic P and Ca levels (Table III). Representative histological sections are shown in Fig. 1(A). Normal integrity of the articular cartilage was observed in the normal (N) group. Microscopic observation revealed that excess phosphorus caused a decrease in small chondrocytes on the articular cartilage surface. Moreover, excess phosphorus induced loss of cells in the middle layer of the articular cartilage. Figure 1(F) shows the number of cell for each 250 μm^2 on a section of articular cartilage in each sample. Figure 1(B) and (E) shows that the thickness of the articular cartilage layer was decreased slightly by excess phosphorus. The thickness of the articular cartilage was reduced in the C group compared to that in the N group. The relative thickness of the articular cartilage of the CH and Pro-Hyp groups increased to approximately 1.3 times that of the C group [Fig. 1(B)]. At the same time, histological observation and micro-CT analysis showed that the subchondral bone was reduced in thickness and bone mass by excess phosphorus [Fig. 1(B)−(D)]. In particular, the edge of subchondral bone was eroded in response to excess phosphorus, and an abnormal shape was observed for the cartilage after it lost its support base [Fig. 1(B) and (D) arrows]. In contrast, CH and Pro-Hyp mitigated the phosphorus-induced effects. Taken together, CH and Pro-Hyp ameliorated the reduction of chondrocytes and subchondral bone [Fig. 1(A)−(D)]. Moreover, with CH and Pro-Hyp, numerous round chondrocytes were observed compared with N [Fig. 1(A)]. In this study, apoptosis was not observed in the sections prepared at 3 weeks, either by TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis or the DAPI method in this high-phosphorus model (data not shown).

CH REGULATES ATDC5 CELL DIFFERENTIATION

Next, we analyzed whether CH and Pro-Hyp affect the proliferation, maturation and differentiation of ATDC5 cells. The proliferation of pre-chondrocytes was quantified by the MTT assay [Fig. 2(B)] and Mayer's hematoxylin (H) staining on day 1 of culture [Fig. 2(A) upper photos]. Metabolic activity of the CH- or Pro-Hyp-supplemented cells was approximately equal to the activity of the negative control cells in the peptone (Pe)-supplemented medium. The staining areas of glycosaminoglycan were compared by AB staining at pH 1.0. After 35 days of culture, ATDC5 cells showed an approximately 1.6 times ($P = 0.03$) greater staining area of glycosaminoglycan in CH and approximately 2.3 times ($P = 0.002$) greater staining area of glycosaminoglycan in Pro-Hyp compared with in Pe medium [Fig. 2(A) middle upper photos and (C)]. Cells that had differentiated into hypertrophic chondrocytes were quantified by measuring the ALP activity by ALP staining on day 5 of culture [Fig. 2(A) middle lower photos, and (D)]. Moreover, mineralization was quantified by measuring calcium deposits by AR staining on day 35 of culture [Fig. 2(A) lower photos and (E)]. The ALP activity and positive areas seen in AR staining of Pe-supplemented cells as a negative control were approximately equal to those in the normal (N) group. The ALP activity and positive areas seen in AR staining of CH- or Pro-Hyp-supplemented cells were less than half of those in Pe-supplemented cells ($P = 0.001$ and $P < 0.0001$). These results indicate that CH contains a bioactive peptide that suppresses the differentiation of ATDC5 cells into hypertrophic chondrocytes and maintain mature chondrocytes that deposit aggrecan in the extracellular matrix.

IDENTIFICATION OF THE BIOACTIVE PEPTIDE THAT AFFECTS ATDC5 CELL DIFFERENTIATION

ATDC5 cells were cultured in the basic medium and also in media prepared by supplementing the basic medium with 2.5 mM of either proline (Pro), hydroxyproline (Hyp), glycine (Gly), proline and hydroxyproline (Pro and Hyp), proline, hydroxyproline and glycine (Pro and Hyp and Gly), Pro-Hyp or prolyl-hydroxyprolyl−glycine (Pro-Hyp−Gly). The cells were stained with Mayer's hematoxylin and examined by MTT assay on day 1 of culture [Fig. 3(A) upper photo, (B)]. As a result of microscopic observation, the morphology of the cells was not changed by Pro, Hyp, Gly, Pro-Hyp or Pro-Hyp−Gly, and none of these affected proliferation of the cells as shown by MTT assay. Next, the cells were stained for ALP on day 5 of culture [Fig. 3(A) lower photo]. None of the amino acid or peptide supplementations with Gly, Pro, Hyp, Pro and Hyp, Pro and Hyp and Gly, or Pro-Hyp−Gly affected the ALP-stained area of the cultured cells compared to the N group. The ALP activity was decreased only by Pro-Hyp, and the ALP-stained area of ATDC5 cells cultured in the Pro-Hyp-containing medium was one-fifth that of the cells cultured in the basic medium [$P < 0.0001$; Fig. 3(C)].

Next, we studied whether the concentration of Pro-Hyp in the medium correlates inversely with the ALP activity of ATDC5 cells. The ATDC5 cells were cultured in media containing 0, 0.1, 0.5, 1, 2.5, 5 or 10 mM Pro-Hyp for 5 days and then subjected to ALP staining [Fig. 4(A) lower photo]. The ALP activity of ATDC5 cells cultured in 1, 2.5 and 10 mM Pro-Hyp was approximately two-thirds, one-fifth, and one-tenth of that of cells cultured in the basic medium (0 mM Pro-Hyp), respectively [Fig. 4(C)]. That is, increasing the concentration of Pro-Hyp in the culture medium reduced the ALP activity of ATDC5 cells in a concentration-dependent manner. These concentrations, however, did not affect ATDC5 cell proliferation as determined by the MTT assay and Mayer's hematoxylin staining [Fig. 4(A) upper photo, (B)].

THE mRNA LEVELS OF CHONDROCYTE-ASSOCIATED GENES AND PEPTIDE TRANSPORTER GENE IN ATDC5 CELLS

We studied whether Pro-Hyp affects chondrogenesis-specific gene expression. The total RNA was extracted 24 h after addition of Pro and Hyp, Pro-Hyp or Pro-Hyp−Gly to the basic medium, and the mRNA levels were measured by RT-PCR [Fig. 5(A)]. Compared to the N group, the cells cultured in the medium containing Pro-Hyp exhibited approximately two-thirds ($P = 0.001$) and one-tenth ($P < 0.0001$) decreases in *Runx1* and OCL mRNA levels, respectively [Fig. 5(B)]. On the other hand, cells cultured in the medium containing Pro-Hyp exhibited an approximately twofold increase in the *aggrecan* mRNA level [$P = 0.004$; Fig. 5(B)]. The *Runx2*, *Smad2* and *Smad4* mRNA levels were not affected by Pro-Hyp. Next, we confirmed whether mRNA of peptide transporter was expressed to ATDC5 cells. PEPT1 mRNA expression was detected by RT-PCR, but it was not affected by Pro + Hyp, Pro-Hyp or Pro-Hyp−Gly. Conversely, PEPT2 mRNA could not be detected in ATDC5 cells by RT-PCR (data not shown).

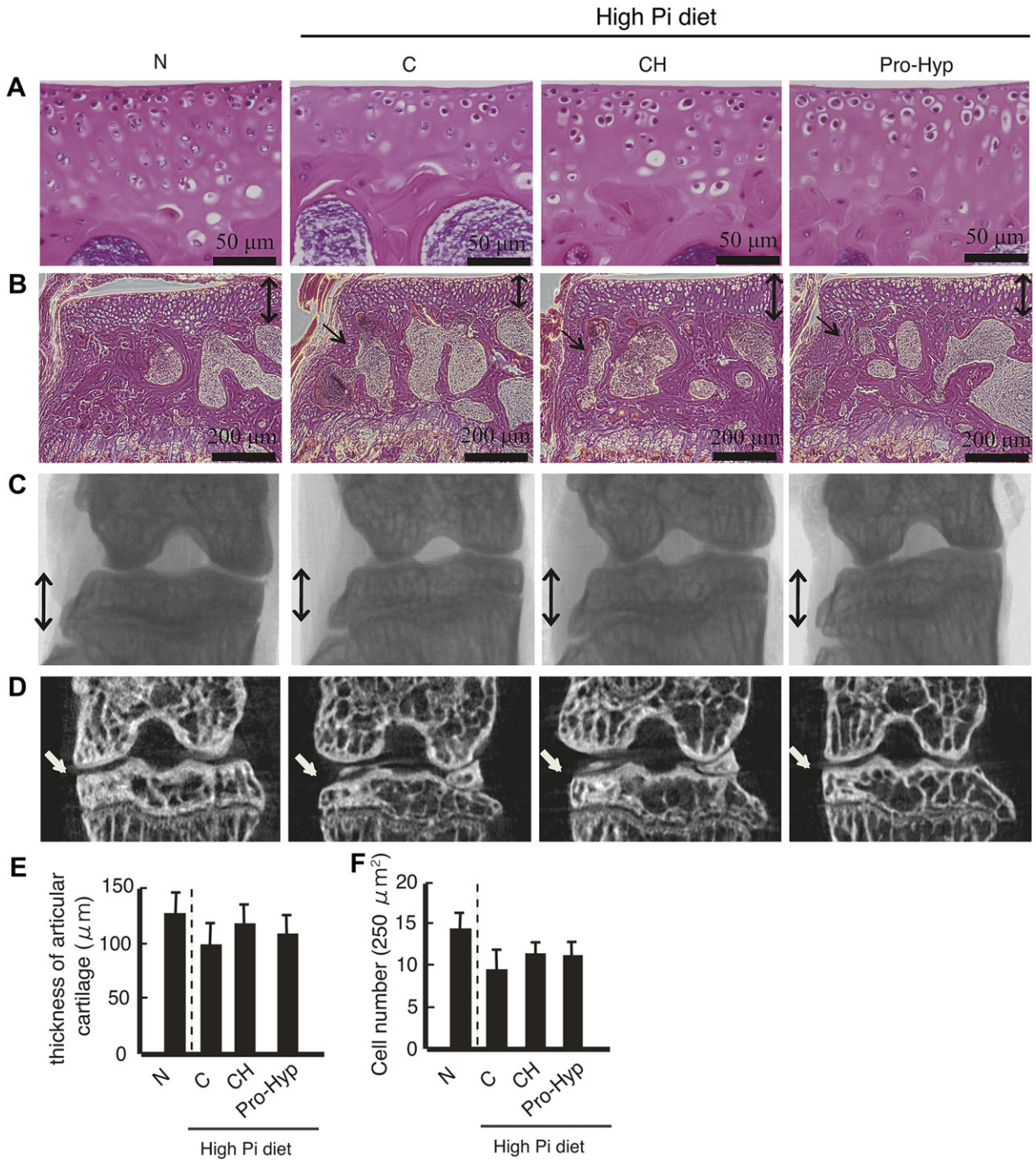

Fig. 1. CH and Pro-Hyp ameliorated the degradation of knee joints in male C57BL/6J mice with phosphorus-induced cartilage degradation. (A) Photograph of high magnification (×400) of histological sections of articular cartilage from the tibial side of the knee. (B) Whole image of histological sections of articular cartilage from the tibial side of the knee (×200). (C) Subchondral bone images prepared by micro-CT analysis. (D) Virtual cross-sections through the bone were reconstructed according to the selected density window using the CT-analyzer software. (E) The thickness of articular cartilage is indicated in the bar graph. The thickness of the articular cartilage was measured at five fixed positions in each mouse. (F) The number of cohndrocyte on sections of articular cartilage is indicated in the bar graph. The number of cohndrocyte was measured at four fixed positions in each mouse. N, C, CH and Pro-Hyp indicate mice that were fed a standard diet, 1.5% phosphorus and 5% gluten as a control diet, 1.5% phosphorus and 5% CH derived from porcine skin, and 1.5% phosphorus and 0.3% Pro-Hyp, respectively.

Discussion

Nutritional factors are important to the maintenance of bone and joint health, and a nutritional imbalance combined with endocrine abnormalities may be involved in the pathogenesis of OA[1,12–14]. High dietary phosphorus intake reduces bone strength and caused soft tissue mineralization[15–17]. A defect in *klotho* gene expression in mice results in articular cartilage degeneration and osteoporosis[18]. Restriction of dietary phosphorus consumption by *klotho* mice

Table III
Serum calcium and phosphorus concentrations

	High Pi diet			
	N	C	CH	Pro-Hyp
Calcium (mg/dl)	10.6 ± 1.7	10.2 ± 1.3	9.6 ± 0.6	11.0 ± 2.0
Phosphorus (mg/dl)	8.5 ± 2.4	8.9 ± 2.7	7.5 ± 1.9	7.7 ± 0.8

Values are mean ± SD; $n = 6$/group.

arrested the cartilage degeneration and osteoporosis[9]. Therefore, it has been suggested that excessive intake of phosphorus accelerates articular cartilage degeneration due to an imbalance in bone and cartilage metabolism. In a preliminary study, we performed observations each week for 4 weeks, and a primary disorder of articular cartilage was noted after 3 weeks on the high-phosphorus diet. Our high-phosphorus diet model is characterized by a primary disorder of articular cartilage due to nutritional imbalance.

There is growing evidence that CH might have beneficial effects on joint conditions[1,2]. However, few studies to date have focused primarily on the effects of treatments on joint conditions[1], and there have been no reports on the mechanism by which CH ameliorates articular cartilage degeneration. Recently, it has been shown that not only amino acids but also oligopeptides are absorbed by the small intestine. Moreover, Pro-Hyp, a dipeptide, was identified as the major constituent of food-derived CH to be detected in human serum and plasma[4]. We hypothesized that Pro-Hyp reaches the articular cartilage and acts as a bioactive peptide, exerting a chondroprotective effect. We studied the effects of CH on articular cartilage *in vivo* from the viewpoint that CH contains Pro-Hyp as a bioactive peptide that ameliorates articular cartilage degeneration.

We predicted that C57BL/6J mice administered CH or Pro-Hyp would show differences in advanced articular cartilage degeneration when excess phosphorus is administered and gluten is used as a negative control. The protein digestibility-corrected amino acid score (PDCAAS) has been adopted by Food and Agriculture Organization/ World Health Organization (FAO/WHO) as the preferred method for the measurement of the protein value in human nutrition. Collagen doesn't contain tryptophan, and PDCAAS of collagen is 0. Gluten shows low PDCAAS value. Therefore, gluten was used for control. Intake of excess phosphorus led to reduced chondrocyte numbers on articular cartilage and decreased density of the cartilage layer and subchondral bone. The serum phosphorus and calcium concentrations were not affected by intake of excess phosphorus. CH and Pro-Hyp suppressed phosphorus-induced degradation of articular cartilage. Moreover, CH and Pro-Hyp caused chondrocytes in articular cartilage to become round.

Next, we used ATDC5 cells for a screening assay to determine the factors that preserve mature chondrocytes. Articular cartilage is thought to preserve mature chondrocytes, keeping them from differentiating into mineralized chondrocytes. In this study, we used the property of ATDC5 cells that they differentiate from prechondrogenic cells into mineralized chondrocytes. We proposed that the factor that prevents differentiation of ATDC5 cells in the mature chondrocyte stage *in vitro* is the same factor that maintains permanent cartilage *in vivo*. Addition of phosphorus to the basic culture medium induced higher mRNA levels for various transcription factors in ATDC5 cells[19,20]. In this study, we experimented with 1 mM phosphorus in the basic medium, since at that concentration it does not induce mRNA levels of transcriptional factors that lead to mineralization.

Our *in vitro* study revealed that CH and Pro-Hyp did not affect ATDC5 cell proliferation, but it remarkably inhibited the ALP activity and showed a larger positive area in AR staining compared with peptone. As for CH, there are a lot of ratios of Pro-Hyp and Pro-Hyp−Gly. Peptone was composed of

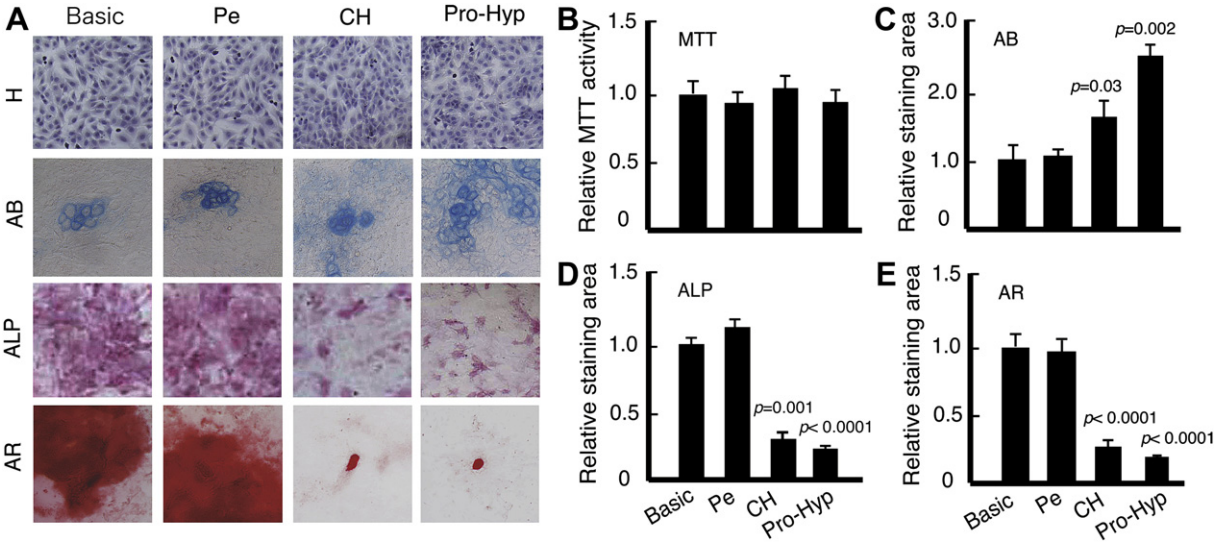

Fig. 2. Effects of CH and Pro-Hyp on the maturation, differentiation and mineralization of chondrocytes (ATDC5 cells). (A) Mayer's hematoxylin (H), AB pH 1.0, ALP and AR stainings were performed to investigate whether CH and Pro-Hyp affect the proliferation, maturation, differentiation and mineralization of ATDC5 cells. (B) The signal intensity of the proliferation of the cells on day 1 was determined by the MTT assay. The signal intensity of the AB-stained cultures on day 35 of culture (C), the ALP-stained cultures on day 5 of culture (D), and the AR-stained cultures on day 35 of culture (E) were determined by densitometry and expressed as bar graphs. (Basic): culture in the basic medium; (Pe): culture in a medium containing 1 mg/ml peptone as the negative control medium; (CH): culture in a medium containing 1 mg/ml CH derived from porcine skin; and (Pro-Hyp): culture in a medium containing 2.5 mM Pro-Hyp. Experiments were carried out in five wells for each medium. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

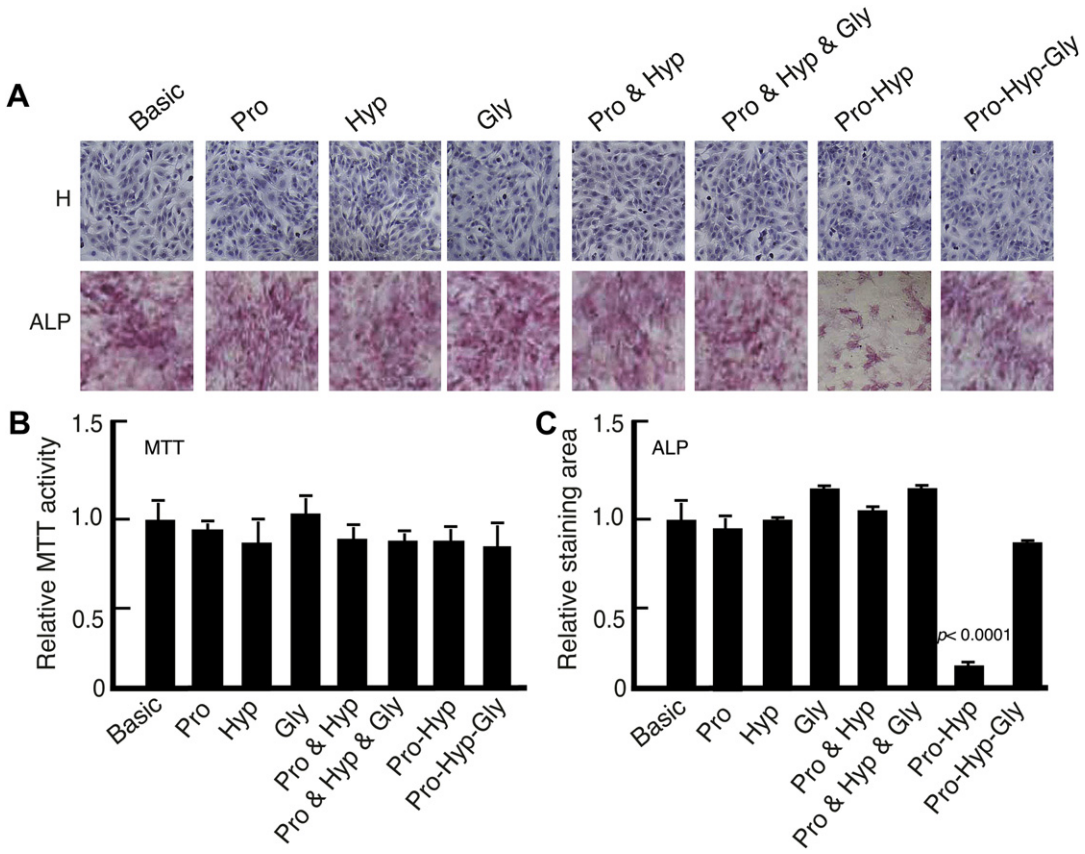

Fig. 3. Effect of Pro-Hyp on ALP activity of ATDC5 cells. (A) The effects of amino acids and peptides on Mayer's hematoxylin-stained area (H) and ALP-stained area. Pro-Hyp and Pro-Hyp−Gly were tested as candidate bioactive peptides. Gly, Pro and Hyp were used as negative controls. Pro and Hyp: addition of both Pro and Hyp to the basic medium; and Pro and Hyp and Gly: addition of Pro, Hyp and Gly to the basic medium. Each peptide and amino acid was added to the basic medium at 2.5 mM. (B) Graph shows the signal intensity of the MTT assay when the cells were cultured with each peptide and amino acid. (C) Graph shows the signal intensity of the ALP-stained area when the cells were cultured with each peptide and amino acid. Experiments were carried out in five wells for each medium. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

various different peptides without specific bioactivity, and thus we used it as a control, avoiding possible effects of specific peptides involved. Moreover, CH and Pro-Hyp induced increased amounts of glycosaminoglycan in the extracellular matrix. Because Pro-Hyp induced increased mRNA levels for aggrecan (Fig. 5), Pro-Hyp might induce chondroitin sulfate. Induction of glycosaminoglycan synthesis in response to Pro-Hyp should be confirmed by biochemical methods such as dithiothreitol (DTT) or 35S incorporation in the future. ALP activity increases at the stage of prehypertrophic chondrocytes. AR staining was used to assess mineralization. Calcification is principally an effect of aging and may contribute to the progression of OA[21–23]. The present data suggest that CH preserves mature chondrocytes and does not allow their differentiation into mineralized chondrocytes.

Many reports have shown that some oligopeptides affect cell function and regulate gene expression *in vivo* and *in vitro*. For example, Pro-Hyp−Gly, Pro-Hyp, etc., stimulate chemotactic activity of fibroblasts, peripheral blood neutrophils and monocytes[24,25]. Asp−Gly−Glu−Ala stimulates osteoblast-related gene expression by bone marrow cells[6]. These reports suggest that some oligopeptides function as bioactive peptides and regulate the function of tissue cells *in vivo*.

The present study found that although such amino acids as glycine, proline, hydroxyproline and Pro-Hyp−Gly did not affect the ALP activity, Pro-Hyp alone caused a reduction in the ALP activity of ATDC5 cells. The basic medium contained about 0.25 mM glycine and proline, but no hydroxyproline. It contained about 1 mM phosphorus, which was not supplemented. In Fig. 3, the final concentrations of Pro, Hyp and Gly were each 2.5 mM. Therefore, amino acids such as glycine, proline and hydroxyproline were not bioactive, but it is surmised that dipeptides such as Pro-Hyp act as bioactive peptides. This suggests that Pro-Hyp might be absorbed in the small intestine after oral administration and that circulating Pro-Hyp might regulate chondrocyte metabolism.

During the process of endochondral ossification, *Runx1* and *Runx2* are coexpressed in limb bud cell condensations that undergo both cartilage and bone differentiation[26–28]. *Runx1* mediates the onset of mesenchymal cell differentiation in chondrogenesis[29]. Another study revealed that the *Runx1* consensus sequence binds to an osteoblast-specific complex and transcriptionally activates the *OCL* gene[27]. In our study, Pro-Hyp regulated the mRNA levels for *Runx1*, *OCL* and *aggrecan* in ATDC5 cells. On the other hand, such free amino acids and tripeptides such as proline, hydroxyproline and Pro-Hyp−Gly did not affect those mRNA levels. The H^+/peptide transporter *PEPT1* and *PEPT2* play a key role in the maintenance of mammalian protein nutrition[30–32]. ATDC5 cells expressed the mRNA of one

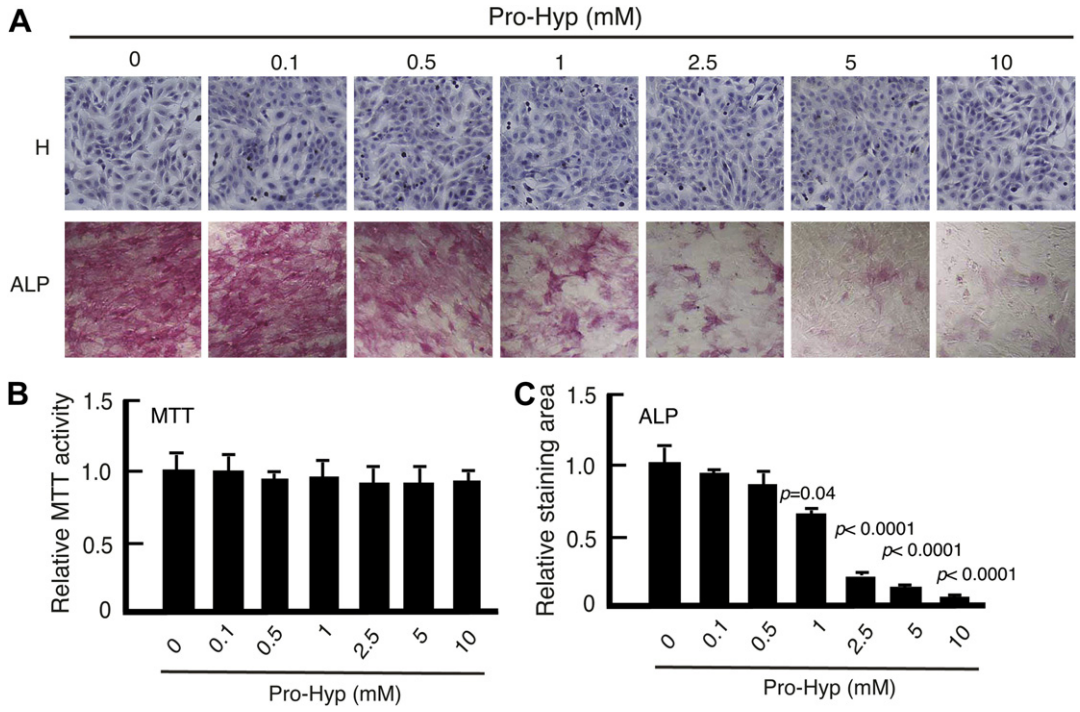

Fig. 4. Concentration-dependent changes in the intensity of the ALP-stained area due to Pro-Hyp. (A) The effects on proliferation and differentiation of ATDC5 cells following the increase in Pro-Hyp concentration were determined by Mayer's hematoxylin staining and ALP staining. (B) Graph shows the relative signal intensity of the MTT assay of Pro-Hyp. (C) Graph shows the relative signal intensity of the ALP-stained area at each concentration of Pro-Hyp. Experiments were carried out in five culture wells. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

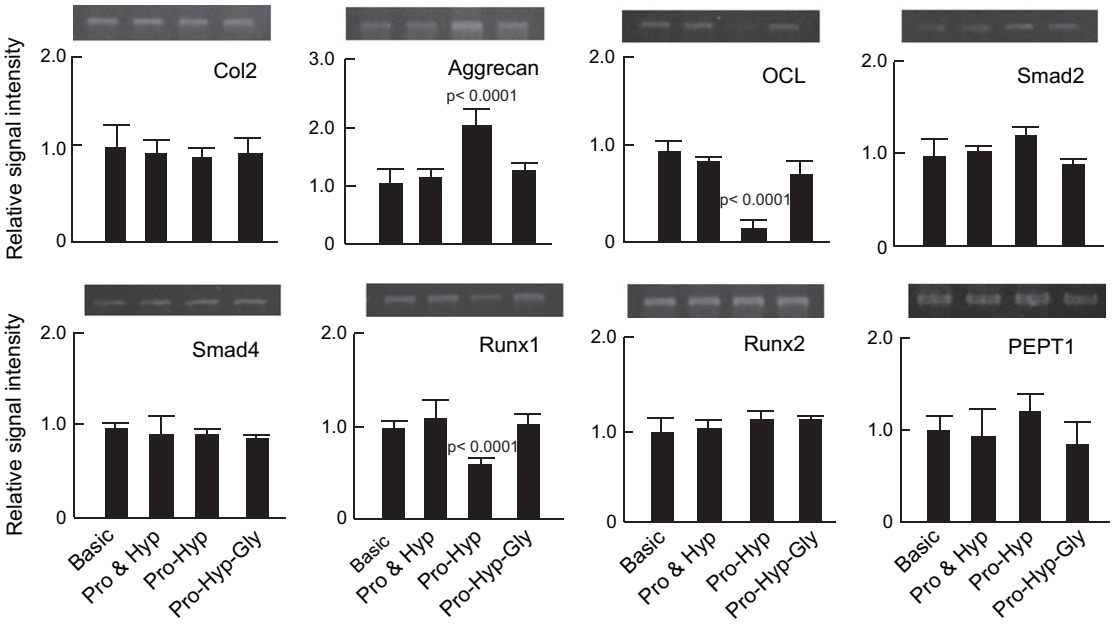

Fig. 5. Effects of Pro-Hyp on mRNA levels for aggrecan, *Runx1*, OCL and PEPT1 in ATDC5 cells. RT-PCR shows that the aggrecan, *Runx1* and OCL mRNA levels were affected by Pro-Hyp in ATDC5 cells. Graphs shows the band intensities determined using ImageJ software, which were obtained from at least three different experiments. The signals were normalized against those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts at 24 h. Experiments were carried out in five culture wells. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t* test. Statistical significance: cells cultured in each medium were compared to cells cultured in the basic medium.

oligopeptide transporter, *PEPT1*, but not *PEPT2*. Therefore, Pro-Hyp is predicted to have a specific binding site for a receptor or intracellular protein, by which it regulates a specific signaling pathway. Consequently, Pro-Hyp might suppress the differentiation to mineralized chondrocytes by repressing the mRNA levels for *Runx1* and *OCL*, and enhancing the mRNA level for *aggrecan*. In the future it will be important to identify the signaling pathway of Pro-Hyp.

Our findings suggest that the Pro-Hyp dipeptide is an important factor that regulates chondrocyte differentiation and plays a role in the maintenance of mature chondrocytes in permanent cartilage. This regulation mechanism by Pro-Hyp suggests that it might be recognized by chondrocytes, leading to reduction in the mRNA level for *OCL via* regulation of the *Runx1* mRNA level and termination of differentiation at the mature chondrocyte stage. Pro-Hyp is present in CH, and this postulated regulation by Pro-Hyp would seem to explain the mechanism of the therapeutic effect of CH in improving joint conditions. It was reported that Pro-Hyp is excreted in human urine[33]. For that reason, Pro-Hyp is formed in the body as a collagen degradation product. This study implies that the bioactive peptide, Pro-Hyp, is derived not only from collagen in living tissues but also from dietary supplements such as gelatin, and that it functions in target tissues.

Further studies are necessary to investigate the effects of CH supplements. Also, understanding the mechanism of action of CH on chondrocyte differentiation would provide a rational basis for the development of chondroprotective therapies for damaged joints.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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