Expression of Calcitonin Gene-Related Peptide Induces Ligament Degeneration Through Endochondral Ossification in Osteoarthritis

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Abstract

Background

Degeneration occurs in various tissues such as cartilage and subchondral bone, and ligament deficiency also plays an important role in the osteoarthritis (OA) progression, but factor which it causes is still unclear. Calcitonin gene-related peptide (CGRP), one of the neuropeptides, its expression increases in OA progression. The purpose of this study is to analyze the mechanism of ligament degeneration and the function of CGRP.

Methods

To examine the relationship between ligament degeneration and CGRP expression, human posterior cruciate ligament (PCL) from OA patients and SAMP8 mice were histologically analyzed. The effect of CGRP of human ligament cells on the differentiation was also examined.

Results

In human PCL, CGRP expression increased as degeneration progressed, and decreased in the severe degeneration. CGRP expressed in the chondrocyte like cells with SOX9. In SAMP8 mice, the expression of CGRP increased as OA progression, but it decreased in the severe OA. CGRP up regulated the gene expression of VEGF, SOX9, RUNX2, Col10a1 and MMP13. CGRP also promoted chondrogenesis and osteogenesis in the human ligament cells.

Conclusions

During OA progression, CGRP induces the transdifferentiation from ligament cells to chondrocytes, and promotes endochondral ossification in the ligament. CGRP would be the therapeutic target to prevent the ligament degeneration.

Introduction

Osteoarthritis (OA) is a progressive joint disorder that increases its prevalence annually. Although OA is a common disease encountered in daily clinical practice, the mechanisms of OA initiation and progression have not been completely elucidated. In the pathogenesis of OA, various tissues including articular cartilage, subchondral bone, meniscus, joint capsule and ligament exhibit progressive degenerative changes, which is one of the reasons for the difficult OA treatment [1]. Thus, elucidating the mechanism of degenerative changes in all joint structures is important. However, few studies on the degeneration mechanism of ligaments have been conducted compared with those on the cartilage, bone, and meniscus despite the importance of ligament degeneration in OA pathogenesis. Cushner et al. reported

that forty-seven percent of patients with OA have ligament degeneration, which may cause joint laxity [2]. Levy et al. demonstrated that histological degenerative changes of the posterior cruciate ligament (PCL) were observed before articular cartilage degeneration [3]. The anterior cruciate ligament and PCL insufficiency frequently cause meniscal and cartilage injuries, which can accelerate the progression of OA due to lateral thrust [4]. Elucidation of the mechanism of ligament degeneration would enable to prevent OA progression. Increasing chondrogenic potential in the ligament has been reported to be a key factor in the degeneration process [5]. During the degenerative process of the ligament, chondrogenic cells increase, and ossification finally occurs in the fibroblast-based ligament [6]. This suggests that some factors may induce chondrogenesis and osteogenesis in the ligament during OA pathogenesis.

Neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, are well known to play an important role in OA-related pain in the joint [7]. Neuropeptides have various functions, including bone metabolism and angiogenesis, to maintain homeostasis [8]. In the pathogenesis of OA, excessive expression of neuropeptides in the subchondral bone causes sclerotic changes in the subchondral bone, and subsequently, cartilage degeneration progresses [9, 10]. CGRP is a neuropeptide belonging to the calcitonin superfamily; it consists of 37 amino acids and is released from sensory nerves [11]. CGRP has various functions, including blood vessel dilation, accelerating inflammatory pain, and promotion of bone remodeling by differentiation of bone marrow mesenchymal cells into osteoblasts, stimulation of osteoblast proliferation, and acceleration of bone metabolism by inhibiting osteoclast differentiation [12]. We focused on CGRP in ligament degeneration because CGRP induces osteogenesis, and the sensory nerve extends into the affected joints during OA progression [13]. We hypothesized that the elongated CGRP-positive sensory nerves into the joint might cause increased chondrogenic potential in the ligament, subsequently progressing ligament degeneration. The aim of this study was to analyze the expression pattern and function of CGRP in the ligament cells in OA pathogenesis.

Materials And Methods

Between June 2016 and October 2018, 30 patients with advanced knee OA were treated with total knee arthroplasty (TKA). They consisted of 11 men and 19 women, with a mean age of 73.5 years. Knee joints were classified as Kellgren-Lawrence grades 3 and 4. Patients with a history of ligament injury, malunited fractures, intra-articular steroid injection in the previous 6 months, joint infection, or previous knee surgery were excluded. During surgery, PCL was harvested for histological analysis according to a previous report [14]. This study was approved by the institutional review board and ethics committee of our hospital and was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all patients.

Histological analysis of human ligament

Harvested PCL was fixed in 4% paraformaldehyde and embedded in paraffin. Four-micrometer-thick sections were prepared for histological analysis. Sections were stained with hematoxylin & eosin (HE) and safranin-O/fast green, and histologically graded using a scoring system according to a previous

report [15]. The following categories were examined and scored for each ligament: (1) inflammation in the ligament substance, (2) mucoid degeneration, (3) chondroid metaplasia, (4) cystic changes, and (5) orientation of collagen fibers. Five fields of the PCL were randomly selected and each slide was evaluated at 200X magnification. The histological changes were scored and graded with reference to previous report as follows [15]: 0, no changes; 0.5, minimal changes; 1, mild changes; 2, moderate changes; and 3, severe changes. The highest summed score of ligament degeneration (total score) was 15 if all five histological categories were scored as severe. Furthermore, the total score was classified into three groups: mild (0–5), moderate (6–10), severe (11–15).

Animals

All procedures were performed in accordance with the Guidelines for Animal Experimentation at our university, and with the approval of the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Senescence-accelerated mouse-prone 8 (SAMP8) mice which develop spontaneous joint OA signs that resemble human disease at the ages of 4, 18, and 42 weeks (n = 9 at each time point) were used for the evaluation of the spontaneous OA model. Harvested knee joints fixed in 4% paraformaldehyde were decalcified for 2 weeks in 20% EDTA and, embedded in paraffin. Four-micrometer-thick sagittal sections, where the whole length of the PCL in the knee joint was observed, were prepared for histological analysis. Sections were stained with HE and safranin-O, and graded histologically using OARSI and ligament scores [15, 16].

Immunohistochemical analysis

For immunohistochemical analysis, each section was immunostained using anti-CGRP antibody (1:500 dilution; Abcam, Cambridge, MA), anti-SOX 9 (1:800 dilution; Abcam, Cambridge, MA), anti-MMP13 (1:20 dilution; Neo Markers, Fremont, CA), and anti-CRLR/CGRP1 polyclonal antibody (1:100 dilution; Bioss, Boston, MA, USA). For double immunofluorescence staining of CGRP and SOX9, the anti-CGRP antibody was labeled with FITC using Dojindo Ab-10 Rapid Fluorescein Labeling Kit (Dojindo Laboratories, Kumamoto, Japan), and anti-SOX9 antibody was labeled using the Dojindo Ab-10 Rapid HiLyte Fluor 555 Labeling Kit (Dojindo Laboratories, Kumamoto, Japan) according to the method described in a previous report [17]. DAPI (Dojindo Laboratories, Kumamoto, Japan) solution was used for nuclear staining. For immunohistochemical signals, five fields in the ligament were randomly selected among the areas where ligamentous structures were found; the total number of cells was counted, and the number of SOX9, MMP13, and CGRP positive cells was measured at 200X magnification using ImageJ software (National Institution of Health) according to the method of a previous report [18, 19].

Cell culture

Human ligament cells were obtained from the anterior cruciate ligament resected with TKA, because in TKA the anterior cruciate ligament is removed and therefore more tissue can be harvested. Ligament tissues were placed in 10-cm diameter Petri dishes under sterile conditions and washed five times with

phosphate buffered saline (PBS; WAKO, Osaka, Japan) to remove red blood cells after removing synovial tissues, adipose tissue, and small blood vessels. The tissue was minced into 1–2 mm pieces, and 0.25% type 1 collagenase was added. Tissues were shaken in a water bath at 37°C for 60 min, and then an equal volume of high-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, Missouri) and antibiotics (at a final concentration of 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B; Nacalai Tesque, Kyoto, Japan) was added to stop degradation. The tissue was then filtered through a 200-mesh nylon filter and centrifuged at 400 rpm for 10 min. The supernatant was discarded, and the human ligament cells were suspended in DMEM medium containing 10% FBS and 1% antibiotics. The harvested cells were homogeneous and were further identified as ligament cells by real time polymerase chain reaction (PCR) analysis as they expressed SCX (Hs 03054634_g1). When the cell coverage reached 80%-90%, the cells were passaged. The cells from passages 2 and 3 were seeded onto 24-well plates (BD Falcon, Franklin Lakes, NJ), and incubated with or without 10 nM and 100 nM CGRP receptor agonist (Peptide Research Institute, Inc., Osaka, Japan) or 10 nM and 100 nM CGRP receptor antagonist (BIBN4096, TOCRIS, Bioscience, Bristol, UK) or the same amount of PBS as a control group added to each well twice a week in a humidified 5% CO₂/95% air atmosphere at 37°C (n = 8 each group). After 24 h, 48 h, and 21 days, RNA was extracted for PCR analysis.

Osteogenic differentiation medium (StemPro Osteogenic Differentiation Kit, Life Technologies, Grand Island, NY) and antibiotics with a final concentration of 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Nacalai Tesque) were used. Calcified medium containing 100 nM of CGRP receptor agonist, or the same amount of PBS were added and cultured for 3 weeks. Osteogenesis capacity was evaluated by PCR and staining with 0.5% alizarin red solution (Nacalai Tesque). To induce adipogenesis, the cells were cultured in adipogenic differentiation medium (StemPro adipogenesis differentiation kit, Life Technologies) and antibiotics, to which 100 nM of CGRP receptor agonist or the same amount of PBS was added. After 3 weeks, adipogenic capacity was assessed using PCR and staining with 0.3% oil red-O (SIGMA O-0625; Sigma-Aldrich Corp.). To induce chondrogenesis, 5×10^5 cells were placed in 15-ml polypropylene tubes (BD Falcon) and pelleted by centrifugation at $500 \times g$ for 5 min. The pellets were cultured in chondrogenesis medium (StemPro chondrogenesis differentiation kit, Life Technologies) and antibiotics, to which 100 nM of CGRP receptor agonist or the same amount of PBS was added for 3 weeks. Chondrogenic capacity was evaluated using PCR and histological analysis. For histological analysis, the pellets were embedded in paraffin, cut into 6-µm sections, and stained with safranin-O/fast green.

Real time PCR

RNA was isolated using TRIzol (Life Technologies) for real-time PCR analysis, and proteins were extracted for analysis of alkaline phosphatase activity. Complementary DNA was synthesized using 1 µg of total RNA using the Superscript VLIO kit (Invitrogen) according to the manufacturer's protocol: MMP13 (Hs 00233992_m1), RUNX2 (Hs 00231692_m1), VEGFA (Hs 0090055_m1), SOX9 (Hs 00165814_m1), COL2A1 (Hs 01064869_m1), COL10A1 (Hs 00166657_m1), PPAR γ (Hs 01115513_m1) and

GAPDH(Hs99999905_m1) using TaqMan gene expression assays probes (Life Technologies), and real-time PCR assays were performed. The expression level of each gene was evaluated relative to the expression level of GAPDH. The $\Delta\Delta C_t$ method was used to analyze the real-time PCR data.

Statistical analysis

All results in this study are expressed as mean $\pm$ standard deviation (SD). Comparisons among the three or four groups were performed using the Tukey–Kramer post hoc test, and the Mann–Whitney U test was used to determine the differences between the two groups. Statistical significance was set at $P < 0.05$.

Results

Expression pattern of the CGRP in human PCL

Of the 30 knees with human ligament degeneration, 7 were in the mild group, 12 were in the moderate group, and 11 were in the severe group. The mean histopathologic score of degeneration of the mild group was 3.3 ± 0.3 points (range, 2–4.5), and the ligament was composed of parallel fiber arrangement, and the cells were mostly fibroblasts. In the moderate group, the mean histopathologic score of degeneration was 6.7 ± 1.5 points (range, 5–9), and the number of chondrocyte-like cells with round nuclei increased, and the surrounding tissue was stained with safranin O. In addition, the ligament fibers become wavy, and the parallel fiber arrangement was disrupted. In advanced degeneration, the mean histopathologic score of degeneration was 12.9 ± 1.5 points (range, 10–14), and the number of chondrocyte-like cells increased and the area stained with safranin O stain also increased. In addition, ossification areas were observed in this study.

The number of SOX9-positive cells was significantly higher in the moderate group than in the mild and advanced groups ($p < 0.01$). The number of MMP13-positive cells was significantly higher in the moderate group than in the advanced group ($p < 0.01$). The number of CGRP-positive cells was lower in the mild group, significantly higher in the moderate group than in the other groups ($p < 0.01$), and decreased again in the severe group (Fig. 1a, b). Immunofluorescent analyses revealed that CGRP and SOX9 were co-expressed in the chondrocyte-like cells in ligament (Fig. 1c).

Expression pattern of the CGRP in the PCL of OA model mouse

To investigate longitudinal ligament degeneration, PCL in spontaneous OA mice (SAMP8) was histologically analyzed. As the number of weeks increased, the OARSI and ligament scores significantly increased with articular cartilage degeneration (Fig. 2a, b). HE and safranin-O/fast green staining revealed parallel fiber arrangement and spindle-shaped nuclei. Ligaments were rarely stained with safranin- O at 4 weeks. At 18 weeks, the increased area in the ligament stained with safranin- O stain and the parallel fiber arrangement decreased, and chondrocyte-like cells were observed. At 42 weeks, ligaments stained almost exclusively with safranin- O stain and the parallel fiber structure disappeared,

and the number of chondrocyte-like cells increased; its degeneration was similar to that of human ligament degeneration. The number of CGRP-positive cells was significantly higher at 18 weeks than at 4 weeks and 42 weeks (Fig. 2a, c). Immunohistochemical analysis of the CGRP receptor showed few positive cells at 4 weeks. However, the positive cells in both fibroblasts and chondrocyte-like cells increased at 18 weeks, and they decreased and were expressed only in chondrocyte-like cells at 42 weeks. Immunohistochemical analysis of CGRP and SOX9 showed that the co-expression of CGRP and SOX9 in the chondrocyte-like cells was highest at 18 weeks, and then decreased at 42 weeks (Fig. 3).

Functional analysis of CGRP on ligament in vitro

To investigate the chondrogenic effects of CGRP on ligament cells, the expression of SOX9, RUNX2, COL10A1, VEGF, and MMP13 was evaluated using real-time PCR. SOX9 expression increased in a CGRP dose-dependent manner at all stages and was significantly suppressed by BIBN4096 treatment. Runx2 expression increased in a CGRP dose-dependent manner at all stages and was suppressed by BIBN4096 treatment. Col10a1 expression significantly increased in a CGRP dose-dependent manner at 3 weeks post-treatment and was significantly suppressed by BIBN4096 treatment (Fig. 4). VEGF expression was significantly upregulated at 24 h post-treatment in the CGRP 100 nM group and at 3 weeks post-treatment in the CGRP 10 nM group. MMP13 expression increased in a dose-dependent manner at 3 weeks and was significantly suppressed by BIBN4096 treatment (Fig. 5). Real-time PCR analyses revealed that CGRP has an effect on ligament cells to promote endochondral ossification, which is inhibited by BIBN4096.

The effect of CGRP on chondrogenesis, osteogenesis, and adipogenesis of ligament-derived cells was examined. In chondrogenesis, the diameter of the pellet with CGRP treatment was larger than that without CGRP treatment (Fig. 6a). In addition, the expression of SOX9 and Col2a1 was significantly higher in the pellet with CGRP treatment than in the pellet without CGRP treatment (Fig. 6a). In osteogenesis, alizarin red staining showed more staining in the ligament cells with CGRP treatment (Fig. 6b). In addition, Runx2 expression in the ligament cells with CGRP treatment was significantly higher than that without CGRP treatment (Fig. 6b). In adipogenic differentiation, oil red staining showed no staining of the ligament after CGRP treatment (Fig. 6c). The expression of PPAR γ was significantly lower in the ligament cells treated with CGRP than in those without CGRP treatment (Fig. 6c).

Discussion

This study showed that CGRP is expressed in chondrocyte-like cells in the ligament as degeneration develops, and CGRP induces chondrogenesis and osteogenesis in the ligament during OA progression. Since ligament dysfunction due to degeneration is an important factor in the pathogenesis of OA, elucidating the factors inducing ligament degeneration will lead to the development of new therapeutic strategies. Neuropeptides, including CGRP, have been recognized as therapeutic targets for various diseases, and receptor antagonists or antibodies have been developed as drugs targeting neuropeptides [20]. In addition, CGRP is a potential target to prevent OA progression by administering a CGRP receptor antagonist through inhibition of subchondral bone sclerosis [9]. Therefore, the evidence that neuropeptides play a crucial role in OA pathogenesis could lead to a novel therapeutic strategy for OA.

Ligament degeneration is histologically characterized by disorganization of collagen fiber arrangement and chondroid metaplasia [15, 21, 22]. Degenerating ligaments exhibit fibrocartilaginous regions with collagen type I and II and an increasing distribution of chondrocytes in addition to disorganization of fiber arrangements [2, 23, 24]. Experimental tendon degeneration showed that approximately 5% of the degenerating area has calcific foci with chondrocytes expressing SOX9 [25]. Kumagai et al. demonstrated that Scleraxis-positive cells decreased and SOX9-positive cells increased as ligament degeneration progressed [5]. Thus, chondrogenic differentiation plays a crucial role in ligament degeneration. However, the factors that induce or promote chondrogenic differentiation of the ligament during OA progression have not been elucidated. We focused on CGRP regarding chondrogenic differentiation in ligament degeneration. CGRP has various functions, including pain perception, osteogenesis, and angiogenesis [26]. The sensory nerve around the joint has been reported to elongate into the joint during OA progression, and the neuropeptides released from the sensory nerve then causes pain, inflammation, and subchondral bone sclerosis [27]. CGRP is distributed in the synovium, subchondral bone, cartilage, and meniscus in the joints with OA [28]. In particular, CGRP expression in the subchondral bone increases in destabilization of the medial meniscus (DMM) mice and induces subchondral bone sclerosis [9]. CGRP may also induces ossification of the ligament during the degeneration process. Moreover, CGRP has been reported to be involved in heterotopic ossification [29], and administration of CGRP to the tibialis anterior muscle of mice promotes heterotopic ossification [30].

We examined the effects of CGRP on the differentiation of ligament cells. Furumatsu et al. demonstrated that ligament cells exhibit chondrogenic properties with SOX9 expression in chondrogenic induction medium [31]. Takimoto et al. showed the direct conversion of tenocytes to chondrocytes by SOX9 overexpression [32]. In our study, CGRP upregulated SOX9 expression in pellet cultures during chondrogenesis, and the size of the pellet was enlarged. Furthermore, CGRP promoted the osteogenesis in the ligament cells while it inhibiting adipogenesis. In fact, with the addition of CGRP to the ligament cells, the expression of SOX9 and Runx2 was increased up to 3 weeks, suggesting that CGRP induces chondrogenesis and osteogenesis. At 3 weeks of culture, the expression of Col10a1 and MMP13, which are important factors in endochondral ossification, increased in a dose-dependent manner, indicating that CGRP induced endochondral ossification [33]. VEGF, important factor in endochondral ossification, is also upregulated by CGRP. Hypervascularization in various tissues in the joint, including the ligament, has been reported in the OA joint [35]. Our study revealed that CGRP might play a role in inducing endochondral ossification and angiogenesis in the ligament through VEGF expression. In our study, CGRP expression increased as the degeneration of the ligament progressed, but severe degeneration ligaments did not exhibit a decrease in CGRP expression accompanied by MMP13 expression. It is suspected that CGRP expression by the sensory nerve that elongates from the surrounding joint induces transdifferentiation of the ligament cells to chondrocyte-like cells to promote endochondral ossification [13]. In the severe degeneration ligament, fibroblastic cells were sparsely present, and CGRP was no longer required. CGRP induces VEGF at 24 h after CGRP addition to the ligament cells. CGRP has been reported to induce angiogenesis [26]. CGRP may also induce angiogenesis in the early phase of OA, which may lead to further increased expression of CGRP [35].

This study had several limitations. First, the natural course of ligament degeneration during OA progression could not be observed in human samples. This is an unsolvable problem for histological evaluation, but we compensated for it by stratifying the degree of ligament degeneration. Moreover, SAMP8, which has been established as a spontaneous OA model, was used to evaluate the time course of ligament degeneration in OA progression [34]. Second, the factors that induce the expression of CGRP in the ligament have not been elucidated, although this study explored important role of CGRP in ligament degeneration. However, the factors that induce OA have not been clarified, despite many studies on OA pathogenesis. Finally, the loss of function of CGRP was not examined in vivo in this study. Knockdown of the CGRP prevents ligament degeneration. Nakasa et al. reported that DMM mice which were administered the CGRP receptor antagonist reduced the OARSI score until 8 weeks, which suggested that inhibition of the CGRP expression may lead to prevent ligament degeneration [9]. However, DMM mice are recognized as a traumatic OA model, and whether DMM affects ligament degeneration is unclear. Therefore, it is desirable to examine CGRP knockdown in a spontaneous OA mouse model.

Conclusion

The present study shows that with regard to ligament degeneration, angiogenesis occurs in the early stages, followed by chondrogenesis and ossification via endochondral ossification, and CGRP is deeply involved in this process. Furthermore, CGRP receptor antagonists were shown to have the potential to effectively attenuate OA progression.

Abbreviations

OA
Osteoarthritis
CGRP
Calcitonin gene-related peptide
PCL
posterior cruciate ligament
TKA
Total knee arthroplasty
HE
Hematoxylin & eosin
SAMP8
Senescence-accelerated mouse-prone 8
PBS
Phosphate buffered saline
DMEM
Dulbecco's modified Eagle's medium
FBS

Fetal bovine serum
PCR
Polymerase chain reaction
SD
Standard deviation
DMM
Destabilization of the medial meniscus

Declarations

Ethics approval and consent to participate

This study was approved by the institutional review board and ethics committee of our hospital and was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all patients.

The study was approved by the Ethics Committee of Hiroshima University Hospital, and written consent was obtained from all patients. All procedures were performed in accordance with the Guidelines for Animal Experimentation at our university, and with the approval of the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Dr. MT and TN designed this study, analyzed and interpreted the data, and drafted the manuscript. TN provided the study materials and funding. AN, MI, YI and SM obtained the data, analyzed and interpreted the data, drafting the article. TN and NA contributed to the critical revision of the article. All authors have read and approved the final manuscript.

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Figures

Figure 1

Expression pattern of the calcitonin gene-related peptide (CGRP) in human posterior cruciate ligament (PCL). (a) Expression pattern of SOX9, MMP13 and CGRP with mild, moderate, and severe degeneration in safranin O staining and immunohistochemistry. (b) Percentage of SOX9-, MMP13-, and CGRP-positive cells. * ; $p<0.05$, ** ; $p<0.01$. (c) Safranin O staining and immunohistochemistry of CGRP and SOX9

Figure 2

Expression pattern of the CGRP in the SAMP8 mice at 4, 18 and 42 weeks. (a) Safranin O staining and immunohistochemistry of the CGRP and CGRP receptor (CGRPR). Dashed line; PCL. Arrows; CGRP in the chondrocyte-like cells. Bar; 100 μ m. (b) OARSI and ligament scores. * ; $p<0.05$, ** ; $p<0.01$. (c) Number of CGRP positive cells. ** ; $p<0.01$.

Figure 3

Double staining of the CGRP and Sox9 in the PCL of SAMP8 mice. Bar; 100 μ m.

Figure 4

Gene expression analyses of SOX9, Runx2, and Col10a1 using polymerase chain reaction (PCR). *; $p < 0.05$, **; $p < 0.01$.

Figure 5

Gene expression analyses of VEGF and MMP13 using polymerase chain reaction (PCR). *; p<0.05, **; p<0.01.

Figure 6

Differentiation analyses of the human ligament cells by the CGRP. (a) Chondrogenesis capacity using pellet culture. (b) Osteogenesis evaluated using alizarin red staining and real-time PCR of Runx2. (c) Adipogenesis evaluated using oil red O staining and real-time PCR of PPAR γ .