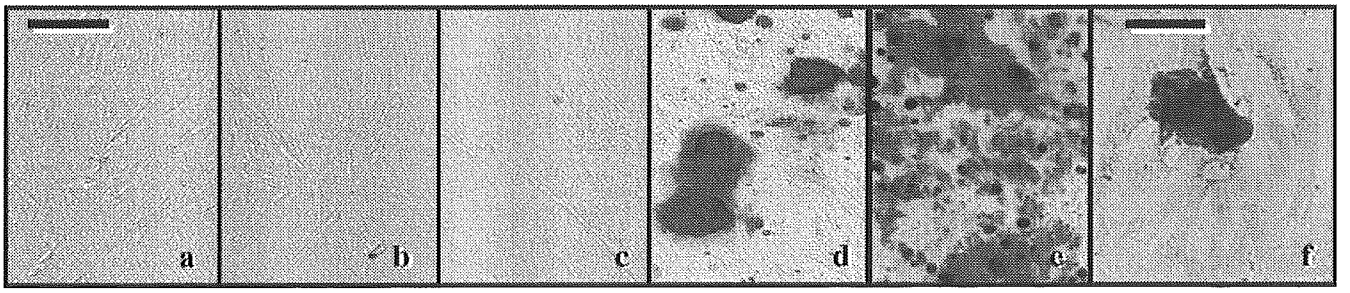


Fig. 6. Mineralization in periodontal ligament subpopulations (PDL-SPs). (a) 30M-PDL-SP, (b) 50M-PDL-SP, (c) 70M-PDL-SP, (d) 90M-PDL-SP, (e) 110M-PDL-SP. The most intensive staining is observed in 110M-PDL-SP and less in 90M-PDL-SP. a–e: ×150, scale bar = 33 μm. Immunoexpression of BSP in 110M-PSL-SP (f). Mineralized nodule is positively stained for BSP. h: ×300, scale bar = 16 μm.

*ALP Activities of PDL subpopulations*

The highest ALP activity was also shown by the quantitative analysis of the enzyme (Fig. 5a) and Fig. 5b shows significant differences between each PDL-SP. As a PDL-SP was derived from farther from the root surface, ALP activity of the subpopulation became less.

*Mineralization*

Well corresponding to the result of ALP activity, different amount of mineralization was seen among the PDL-SPs (Figs. 6a–e). Little or no staining was seen in 30M-, 50M-and 70M-PDL-SPs (Figs. 6a–c). The most intensive staining was observed in 110M-PDL-SP (Fig. 6e) and less in 90M-PDL-SP (Fig. 6d). BSP was immunohistochemically expressed in mineralized nodules and surrounding cells (Fig. 6f).

*Expression of mRNA for mineralization related markers in 30M- and 110M-PDL-SPs*

Although expression of COLI, ALP, OPN and OCN-mRNA was observed in both of 30M-and 110M-PDL-SPs, 110M-PDL-SP expressed higher levels of OPN and OCN-mRNA. In addition, BSP mRNA could be detected only in 110M-PDL-SP (Fig. 7).

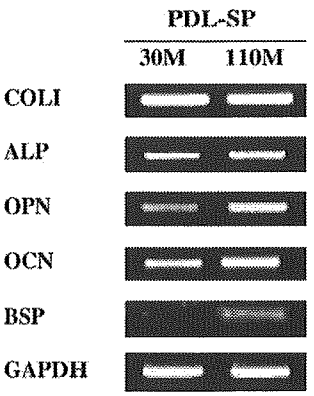


Fig. 7. mRNA-expression of mineralization related markers in 30M- and 110M-PDL-SPs. Expression of COLI, ALP, OPN and OCN mRNA was seen in both of 30M- and 110M-PDL-SPs. BSP-mRNA was only detected in 110M-PDL-SP.

*Immunolocalization of PCNA and BrdU in vivo PDL*

To confirm the high growth potential of 100M-SP-PDL, immunodetection of PCNA in vivo PDL (Fig. 8a) and BrdU incorporation into PDL cells (Fig. 8b) were examined. Positive cells for both proliferation markers were observed near the root surface as well as in the perivascular area in the middle of PDL.

## Discussion

Enzymatic release of PDL cells from extracted roots has been applied in some studies and cellular characteristics of the cells were studied [12,13,16]. In those studies, however, the cells were harvested in the lump and analyzed as a tissue unit. In other words, heterogeneity of PDL has not been examined using subpopulations isolated from PDL by sequential enzymatic digestion, which has been applied to characterize heterogeneous calvarial cell populations [10,11]. Based on previous reports [12,13,16] and our preliminary studies, we decided on a 110 min. digestion to release all cell layers of PDL from extracted roots. Complete release of PDL cells was confirmed at the light and electron microscopic levels. It was also verified histologically that pulpal cells were not released into the

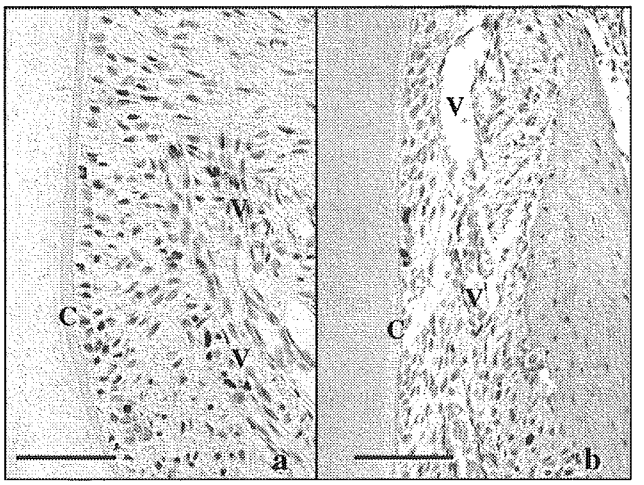


Fig. 8. Immunostaining for proliferating cell nuclear antigen in periodontal ligament (PDL) (a) and BrdU incorporation into PDL cells (b). Positive cells for both markers are observed in PDL cells near the root cementum (C) as well as in the perivascular area (V) in PDL. a: ×200, scale bar = 50 μm, b: ×100, scale bar = 100 μm.

digestions for the experimental period. In the present studies, we obtained 5 PDL-SPs. 30M-PDL-SP was considered to be isolated from the middle part of PDL, because about a half of the width of PDL attached to the extracted roots. Although light and electron microscopy showed a few layers of PDL cells on the root surface after 90 min digestion, those cells were completely disappeared from the root surface after 110 min digestion. Moreover, cementocytes located in mineralized cementum matrix were not released into the digestion without decalcification. We therefore regarded 110M-PDL-SP as a subpopulation enriched by lining cells on the root surface.

Studies on ALP and mineralization activities of the subpopulations showed that higher activities were seen in PDL-SP toward the root surface and that 110M-PDL-SP had by far the highest activity among the subpopulations. Groeneveld et al. [16,17] reported that high ALPase activity is observed in the area near cementum and alveolar bones at rat maxillary molar in vivo. Sasaki et al. [18] also showed that cementoblasts of human deciduous teeth exhibited intense ALP activity along the plasma membranes of whole cell surfaces. In addition, 110M-PDL-SP showed the expression of mineralization related genes, including COLI, ALP, OPN, OCN and BSP. Levels of OPN and OCN-mRNA in 110M-PDL-SP were higher than those in 30M-PDL-SP. BSP-mRNA could not be detected in 30M-PDL-SP. MacNeil et al. demonstrated that BSP, OPN and OCN mRNA were expressed selectively by cells lining the root surface (cementoblasts) during root development in mice. Our results and these findings also suggest that 110M-PDL-SP is enriched cells from the root surface: cementoblasts.

McCulloch et al. [8,9], based on their radioautographic studies of mouse mandibular molar prepared from animals pulse-injected with 3H-Tdr, reported that cells migrate from endosteal spaces into the PDL and they express the phenotypes for osteoblasts or cementoblasts. They showed that, numerous cells labeling with 3H-Tdr, which seemed to be progenitor cells, were observed in the surrounding blood vessel. Well corresponding to their results, 30M-PDL-SP obtained from the middle area of PDL showed a high proliferative potential and considered to be enriched progenitor cells.

PDL-SP gradually decreased its potential of growth as it approached toward the root surface. Interestingly, however, 110M-PDL-SP had a high proliferative activity equivalent to that of 30M-PDL-SP. To confirm the high growth potential of 110M-PDL-SP, immunodetection of proliferating cell nuclear antigen in PDL (Fig. 4a) and BrdU incorporation into PDL cells (Fig. 4b) were examined. Positive cells for both proliferation markers were observed near the root surface as well as in the perivascular area in the middle of PDL. Together with these findings, there are two possibilities that 110M-PDL-SP is a homogeneous cell population with high potentials of both mineralization and proliferation or heterogeneous population composed of cementoblasts with high potential of mineralization and immature cells with high growth potential which can differentiate into cementoblast. The latter is more likely, because cultured 110M-PDL-SP showed uneven staining pattern for ALP which was most intensive around mineralized nodules. The origin of the cells with such a high growth potential on root surface is not decided. During root formation, Hertowig's epithelial root sheath (HERS) disintegrates prior to cementogenesis and undifferentiated mesenchymal cells from dental follicle penetrate HERS to invade the root surface. The undifferentiated cells differentiate to cementoblasts at root surface and deposit initial cementum [19,20]. A potential explanation is that a part of the undifferentiated cells with high proliferative activity migrated from dental follicle took up residence near the root surface after the cementum formation period. Further studies is necessary to determine this unique cell population is existing only in PDL of the 8 week-old animals examined or lasting there to the end of their life. If such an interesting population exists ever since, the population might play great roles especially in periodontal tissue remodeling and regeneration.

Although we could not characterize PDL-SPs in alveolar bone half of PDL, the present studies showed 1. PDL-SPs obtained from the cementum half of PDL by sequential enzymatic digestion showed different activities in proliferation and mineralization. 2. PDL-SPs with higher proliferation rates were generally located in the middle portion of PDL and those with higher mineralization activities were seen toward the surface of the roots. 3. PDL-SP on the root surface showed high activities of proliferation and mineralization. It is suggested that a possible pathway of PDL cell turnover may exist within the PDL-SP on the root surface in addition to the generally recognized pathway from the middle area of PDL to root surface. To clarify the mechanism of cellular proliferation and differentiation in PDL using PDL-SPs will provide indispensable data for developing new regenerative periodontal therapies. There is a possibility that the implantation of isolated root lining cell population having the high potential both of proliferation and minertalization may bring the adequate formation of cementum, which is needed for regeneration of periodontal tissue lost due to the periodontal disease.

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ORIGINAL PAPER

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# Vascular endothelial growth factor plays an important autocrine/paracrine role in the progression of osteoarthritis

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**Abstract** Vascular endothelial growth factor (VEGF) plays an essential role in the angiogenesis of growing cartilage. Although VEGF expression in cartilage vanishes in normal adults, VEGF is known to be expressed in chondrocytes of osteoarthritic (OA) cartilage. As little information is available on the VEGF expression in the cartilage of OA-like lesions of the temporomandibular joint (TMJ), VEGF expression in the condylar cartilage of TMJs of rats affected with OA was examined. To evoke OA, mechanical stress was applied by forced jaw opening for 10 or 20 days. After 20 days, marked OA-like lesions were observed in the condyle. VEGF was expressed in the chondrocytes of the mature and hypertrophic cell layers of the intermediate and posterior region of the condyle. The percentage of VEGF immunopositive chondrocytes significantly increased with the period of applied mechanical stress. Furthermore, tartrate-resistant acid phosphatase (TRAP) staining of the condylar cartilage showed significant increment of osteoclasts in the mineralized layer subjacent to the hypertrophic layer where high VEGF expression could be detected. The results suggest that VEGF plays an important role in the progression of OA.

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## Introduction

Articular cartilage is an avascular tissue, which produces angiogenic inhibitors (Horner et al. 2001). The hypertrophic cartilage layer, however, acts as a target for capillary invasion and angiogenesis with synthesis of angiogenic activators (Descalzi et al. 1995; Alini et al. 1996). Angiogenesis is considered to be essential for replacement of cartilage by bone during skeletal growth and regeneration (Gerber et al. 1999). Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with specific mitogenic and chemotactic actions on endothelial cells (Garcia-Ramirez et al. 2000). VEGF is produced by the chondrocytes of the hypertrophic cartilage layer, and together with its receptors (Flt-1 and Flk-1), it plays an essential role in both embryonic vasculogenesis and angiogenesis during normal growth (Gerber et al. 1999; Horner et al. 1999; Pufe et al. 2001; Aoyama et al. 2004). Although these VEGF-induced processes discontinue after the growth period, the presence of VEGF most likely continues to be required for active angiogenesis in processes such as tissue remodeling and wound healing and may play a role in malignant or certain inflammatory diseases of the adult (Neufeld et al. 1999). VEGF is also produced in chondrocytes of cartilage in joints affected with osteoarthritis (OA) (Neufeld et al. 1999; Enomoto et al. 2003). VEGF could have a pivotal role in the formation of new vessels in OA cartilage (Radin and Paul 1971). Indeed, osteophyte formation and extension of the subchondral growth plate have been observed in OA. These processes are very similar to enchondral ossification, which is highly dependent on VEGF (Gerber et al. 1999).

In the temporomandibular joint (TMJ), OA can be caused by a variety of mechanical and/or inflammatory factors. Previous studies have demonstrated VEGF expression in affected TMJ discs. This expression correlates with formation of new vessels in the avascular portions of the disc as well as with the prevalence of

chondroblast- and fibroblast-like cells (Leonardi et al. 2003). Little information is, however, available on the expression of VEGF in the condylar cartilage of the osteoarthritic TMJ. We tested the following hypotheses: (1) OA-like lesions can be induced by repeated mechanical overloads in a time-dependent manner, and (2) the expression and localization of VEGF in the condylar cartilage can be associated with mechanical overloading.

## Materials and methods

### Experimental animals

Fifteen 9-week-old Wistar strain male rats were used in this investigation. They were fed a stock diet with water ad libitum. The animals were divided into three groups of five. In two experimental groups, the TMJ was repetitively overloaded during a period of, respectively, 10 or 20 days; the third group served as an untreated (control) group. All procedures performed in this experiment were approved by the Hiroshima University Animal Care and Use Committee.

### Application of mechanical stress

In the experimental animals, mechanical stress was applied to both TMJs by applying extended mouth opening for 1 h/day by using a jaw-opening device. This device kept the mandible in a maximal mouth-opening position of 30 mm. During this period of joint overload, the rats were anesthetized with intraabdominal injections of sodium pentobarbital (Nembutal; Dinabott, Osaka, Japan) at a dose of 50 mg/kg body weight, This procedure was repeated for 10 or 20 consecutive days. In the control animals, no mouth opening was applied although the same anesthesia schedule was maintained.

### Tissue preparation

After the experimental period, the animals were killed with an overdose of anesthesia. The animals of the control group were killed after 10 days. Both TMJs were resected and fixed in 10% buffered paraformaldehyde and decalcified with 10% EDTA for 4 weeks at 4°C. Thereafter, they were embedded in paraffin, and serial sections (7 μm) were cut in the sagittal plane. The sections were stained with hematoxylin and eosin (HE) for histological examination.

### Immunohistochemistry

To investigate the expression of VEGF in the condylar cartilage, immunohistochemical staining was performed using an anti-VEGF antibody (Immuno Biological Laboratories, Fujioka, Japan). Immunostaining was carried out using a Histofine simple stain kit (Nichirei, Tokyo, Japan). Briefly, after blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, nonspecific binding of the antibody was blocked by incubating the section for 30 min with nonspecific staining blocking reagent (Dako, Carpinteria, CA, USA). Thereafter, it was incubated with the VEGF antibody (20 μg/ml in PBS) at 4°C overnight in a humid atmosphere. After washing, the sections were incubated with goat antirabbit IgG (Fab-fragment) conjugated to peroxidase-labeled amino acid polymer for 45 min. Color was developed with 0.02% 3,3′-diaminobenzidine (DAB) and 0.006% $H_2O_2$ in 0.05 M Tris–HCl buffer (pH 7.6). The sections were then dehydrated, mounted, and counterstained with Mayer's hematoxylin. The localization of VEGF-positive cells in the condylar cartilage was microscopically examined. Staining specificity was ascertained by substitution of the primary antibody with PBS or 3% normal rabbit serum.

### Tartrate-resistant acid phosphatase (TRAP) staining

Tartrate-resistant acid phosphatase (TRAP) activity was examined for characteristics of osteoclast lineage cells according to the method of Minkin (1982). The staining medium contained naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO, USA) as substrate, Fast red violet LB salt (Sigma) as the coupler, and 50 mM sodium tartrate (Wako, Osaka, Japan). Counterstaining was performed with hematoxylin. Negative staining was performed without substrate.

### Histometric analysis

From each TMJ, we selected one section from the midsagittal plane of the condyle. The condylar cartilage in each section was divided into anterior, intermediate, and posterior regions, as they are anatomically and functionally distinct areas. Furthermore, in each region, the condylar cartilage was divided into four layers (fibrous layer, proliferating cell layer, mature cell layer, hypertrophic cell layer). In each of these 12 areas (three regions × four layers), the number of VEGF-positive and -negative chondrocytes was counted under a fixed measuring frame (450 μm × 900 μm); the percentage of VEGF-positive cells was calculated as the number of VEGF-positive cells per the total number of chondrocytes under a fixed measuring frame. All chondrocytes in which cytoplasm was distinctively stained were considered to be VEGF positive. Using one section with TRAP staining through the midsagittal plane of the condyle, the number of osteoclasts was also counted in the mineralized layer subjacent to the hypertrophic cell layer of condylar cartilage. TRAP-positive cells with two or more nuclei were counted as osteoclasts. For each animal, the mean of both TMJs was calculated. For each of

the three groups, mean ± SD values were determined. We used Scheffe's test to check for differences in the numbers of total cell, VEGF-positive cell, and TRAP-positive osteoclasts between the experimental and control animals. Probabilities of less than 0.05 were considered to be significant.

## Results

### Histological and immunohistochemical findings

The condyles from the control animals showed smooth articular surfaces without signs of OA (Fig. 1A, C). Their four different layers could easily be distinguished, and the cells were regularly arranged. In addition, the disc contained many cells, and its collagen fibers were running regularly (Fig. 1B); the disc showed no degenerative changes.

The condylar cartilage of the animals that had experienced joint overload for 10 days (the 10-day group) had a normal appearance, with the exception of one joint that showed an irregular cell alignment in the posterior region of the condyle (Fig. 1D–F). In the 20-day group, marked OA-like lesions were observed in the condylar cartilage (Fig. 1G–I), including decrease in the thickness of the cartilage layer, irregularities of chondrocyte alignment, compressive necrosis of chondrocytes, and hyalinization of cartilage matrix in the proliferating, mature, and hypertrophic cell layers. In addition, increase of blood vessel and multinucleated osteoclasts was observed in the area subjacent to the hypertrophic cell layer. These changes were mainly detected in the intermediate and posterior region of the condyle. Furthermore, the disc showed extensive degenerative alterations, such as thinning, hyalinization, and cell-free areas (Fig. 1H).

In the control group, very few VEGF-positive cells could be detected in any of the cartilage layers (Fig. 2A). In the 10-day group, VEGF was abundantly expressed in the chondrocytes located in the mature and hypertrophic cell layer of the intermediate and posterior region of the condyle (Fig. 2B). In the 20-day group, VEGF expression of chondrocytes in the mature and hypertrophic cell layers was enhanced with respect to the 10-day group (Fig. 2C). Chondrocytes near necrotic areas showed prominent VEGF expression.

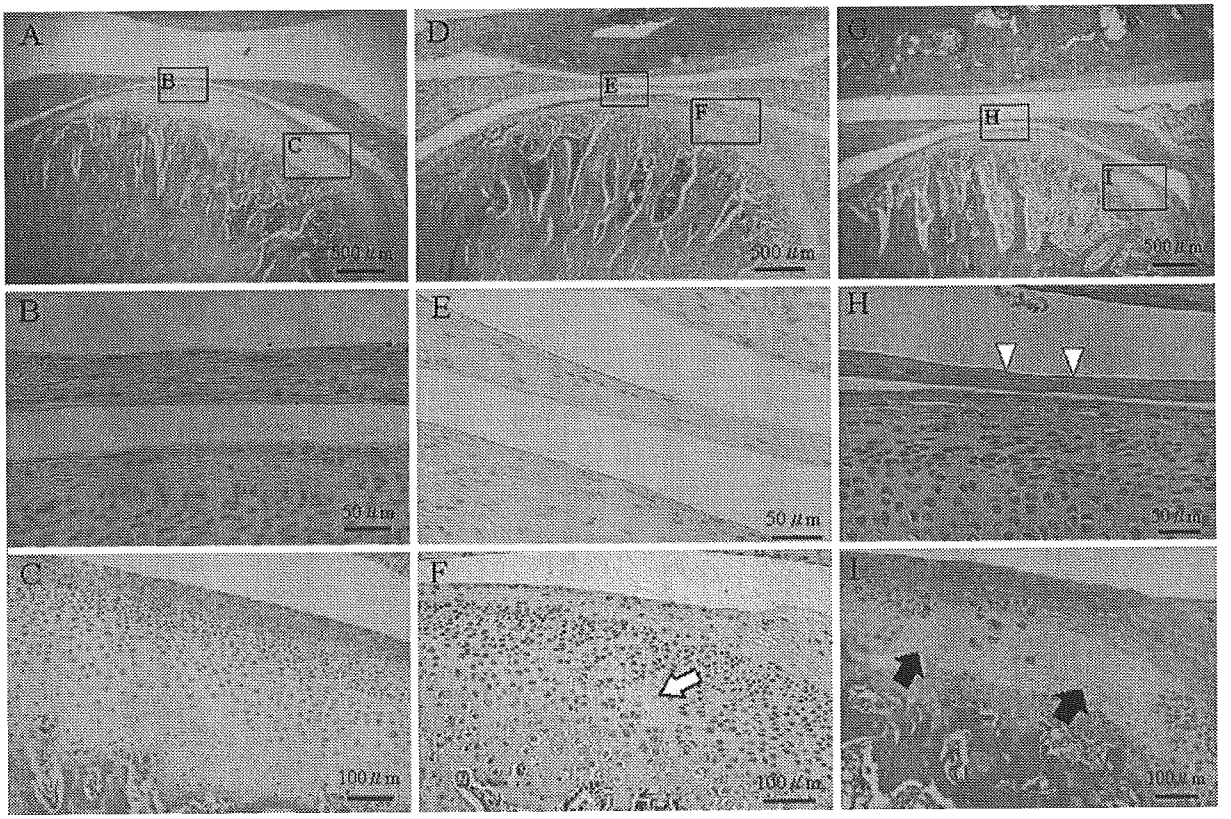


**Fig. 1** Hematoxylin and eosin (HE)-stained sections of the rat temporomandibular joint (TMJ) after stress application. **A** Control TMJ. **B, C** Higher magnification of the area within the rectangular frame placed on *panel A*. **D** Experimental TMJ after 10 days' jaw opening. **E, F** Higher magnitude of the area within the rectangular frame placed on *panel D*. Condylar cartilage appeared to be normal except for one condyle with an irregular cell alignment in the posterior region (*white arrow*, *panel F*). **G** Experimental TMJ after 20 days' jaw opening. **H, I** Higher magnitude of the area within the rectangular frame placed on *panel G*. Osteoarthritis-like lesions were observed in the condylar cartilage. The articular disc showed thinning, hyalinization, and cell-free areas (*white arrowhead*, *panel H*). The condylar cartilage showed irregularities of chondrocyte alignment, pressure death of chondrocytes, and extensive hyalinization (*black arrow*, *panel I*)

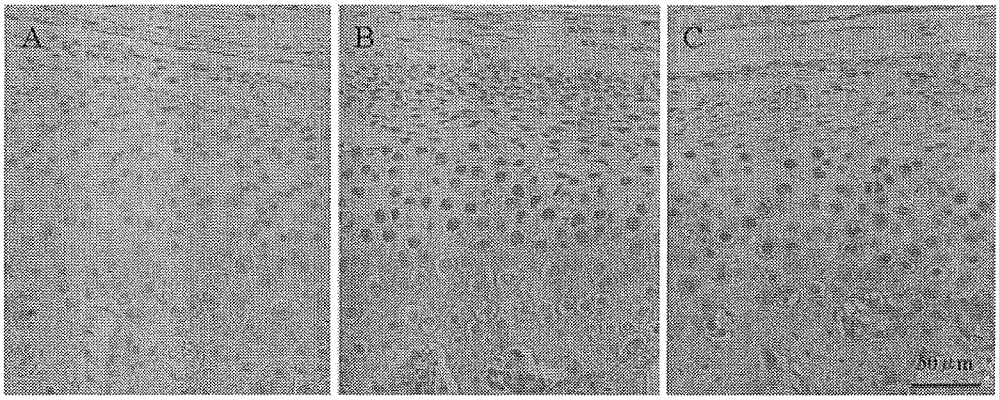


**Fig. 2** Immunohistochemical staining of vascular endothelial growth factor (VEGF) in the condylar cartilage. **A** Control group. **B** Ten-day group. **C** Twenty-day group. VEGF-positive cells (*brown, panel B and C*) were detected only in the mature and hypertrophic cell layers

In the control group, small number of TRAP-positive osteoclasts was seen in the mineralized layer (Fig. 3A). Mechanical stress applied by forced jaw opening induced obvious increase in number of osteoclasts in the mineralized layer subjacent to mature and hypertrophic cell layers where VEGF expression was upregulated (Fig. 3B, C).

## Histometric findings

### *Total cell number*

In the control group, the total number of chondrocytes in the midsagittal section of the condylar cartilage was $191 \pm 19$, $208 \pm 10$, and $316 \pm 28$ (mean ± SD) at the anterior, intermediate, and posterior regions, respectively (Fig. 4). In all three regions, the proliferating cell layer contained the largest amount of chondrocytes. There were no significant differences in the number of chondrocytes between the control and experimental groups in the anterior region. In the intermediate and posterior region, the total number of chondrocytes was significantly smaller ($P < 0.05$) in the 20-day group than in the 10-day group. The difference in cell number was largest in the posterior region where the number of chondrocytes in the proliferating and mature cell layers was significantly less in the 20-day group ($P < 0.05$).

### *The number of VEGF-positive cells*

The average amount of VEGF-positive cells in the cartilage of the condyle of the control group was 9.6%. This amount increased significantly ($P < 0.05$) to more than 40% in both experimental groups (Fig. 5). In the 10-day group, this increase was predominantly observed in the mature and hypertrophic cell layer, with a percentage of VEGF-positive cells of 79.1% and 79.5%, respectively; these percentages were significantly larger than those in the control group ($P < 0.05$). In the 20-day group, the increase of VEGF-positive cells compared with the

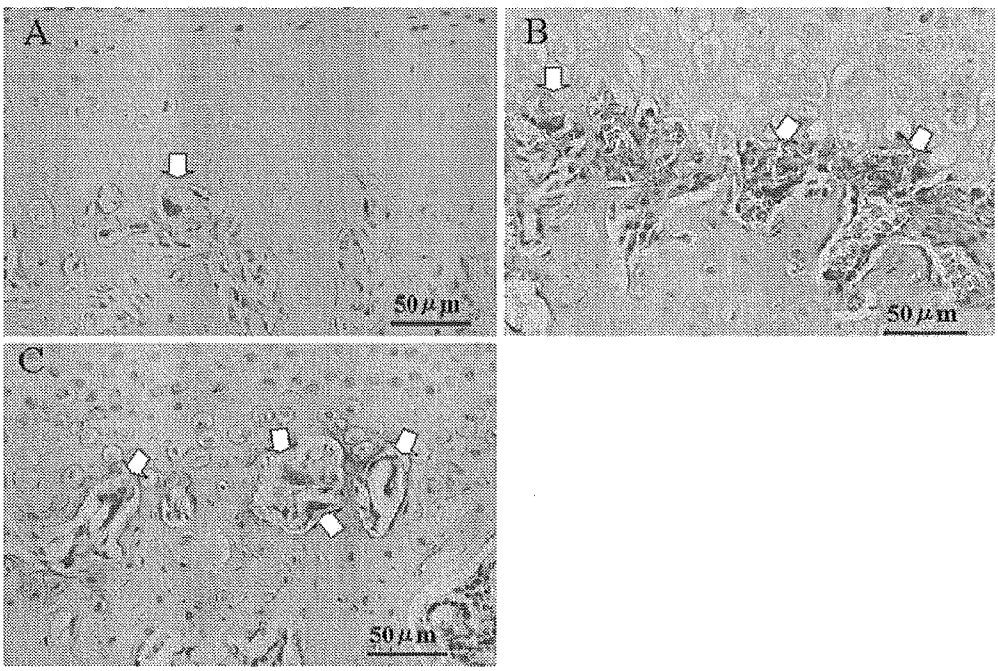


**Fig. 3** Tartrate-resistant acid phosphatase (TRAP) staining in the mineralized layer subjacent to the hypertrophic cell layer of condylar cartilage. **A** Control group. **B** Ten-day group. **C** Twenty-day group. *White arrows* indicate osteoclasts

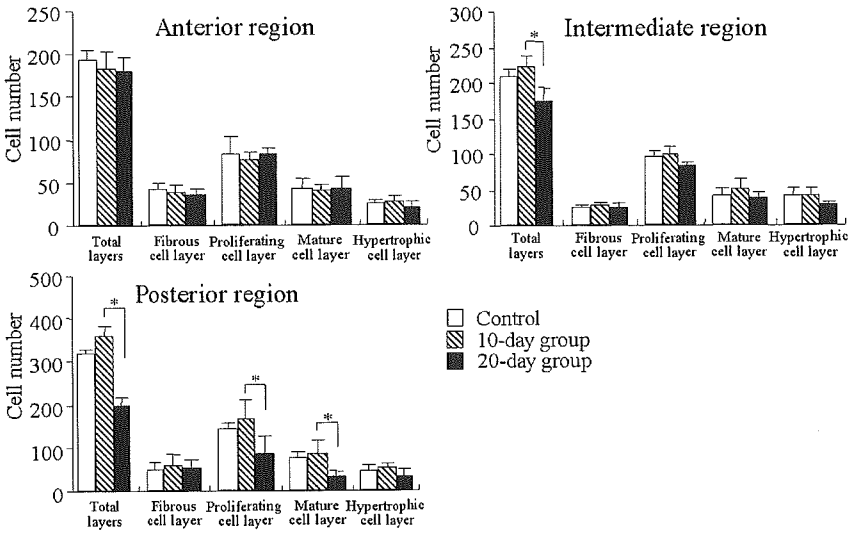


**Fig. 4** Number of chondrocytes in the various regions and layers of the condylar cartilage. The *asterisk* indicates $P < 0.05$ using a Scheffe's test

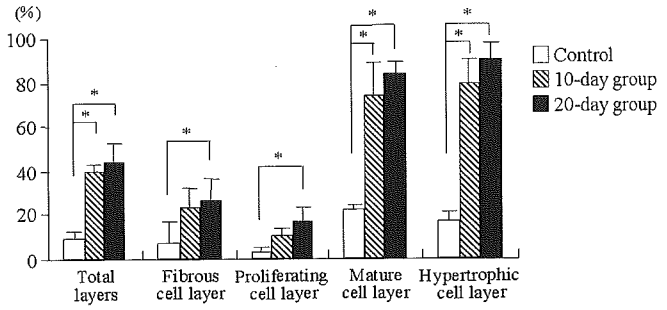


**Fig. 5** Percentage of vascular endothelial growth factor (VEGF)-positive cells in the condylar cartilage. The *asterisk* indicates $P < 0.05$ using a Scheffe's test

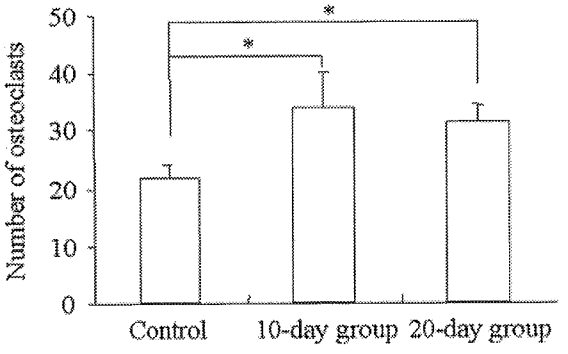


**Fig. 6** Number of osteoclasts in the mineralized layer subjacent to the hypertrophic cell layer of condylar cartilage. The *asterisk* indicates $P < 0.05$ using a Scheffe's test

control group was significant ($P < 0.05$) in all cartilage layers.

### *The number of TRAP-positive osteoclasts*

In the control group, the number of osteoclasts detected in the mineralized layer was $22.0 \pm 2.0$ (Fig. 6). After a forced mouth opening, the numbers of TRAP-positive osteoclasts were $34.0 \pm 5.8$ in the 10-day group and $31.5 \pm 3.1$ in the 20-day group. There were significant differences in the number of osteoclasts between the control and experimental groups ($P < 0.05$).

## Discussion

In growing articular cartilage, hypertrophic chondrocytes promote vascularization in the hypertrophic cell layer in the growth plate by production of VEGF, but this process discontinues in the adult (Gerber et al. 1999). Furthermore, normal chondrocytes secrete antiangiogenic peptides and inhibitors of proteases. As these constituents are involved in extracellular matrix degradation (Moses et al. 1999), they must likely inhibit angiogenesis, which can explain why articular cartilage is normally an avascular tissue. Conversely, in OA cartilage, a focal premature differentiation of chondrocytes to hypertrophic cells was demonstrated (von der Mark et al. 1992). These hypertrophic cells produce VEGF, which may contribute to the inflammatory process in OA. It is likely that similar processes occur during the development of OA in the TMJ as a result of mechanical stress. However, in contrast to the many reports on the role of VEGF in inflammatory arthritis, such as rheumatoid arthritis, there are few reports on its role in OA in the TMJ, despite its frequent occurrence. The present study is, as far as we know, the first in which the VEGF expression in the condylar cartilage of TMJ-OA has been examined.

Unlike rheumatoid arthritis and synovitis, TMJ-OA has primarily a noninflammatory origin (Zarb and Carlsson 1995). The pathological process is characterized by deterioration and abrasion of articular cartilage and local thickening and remodeling of the underlying bone (Zarb and Carlsson 1995). These changes are frequently accompanied by the superimposition of secondary inflammatory changes. Therefore, mechanically induced OA may better reflect TMJ-OA. Recently,

Fujisawa et al. (2003) reported on the possibility of developing OA-like lesions in the rabbit TMJ by forced mouth opening. Their experimental protocol included a repetitive, steady, mouth opening of 3 h/day for 5 days. Muto et al. (1995) investigated pathological changes of the rat TMJ induced by repeated mouth opening of 20 mm ten times per day for 10 days. Although these authors reported the occurrence of synovitis and fibrous adhesions, it would not be appropriate to call these findings OA. Considering these findings, we used a protocol of a forced mouth opening of 30 mm for 1 h/day. With this protocol, OA-like lesions in the TMJ could be induced that were similar to those observed in TMJ-OA patients. After 20 days of forced mouth opening, marked OA-like lesions could be recognized. Therefore, our protocol of forced mouth opening can be considered as a useful model to evaluate the initiation and advancement of OA.

The VEGF expression in OA cartilage appeared to be progressive with the applied mechanical overload. Freemont et al. (1997) reported that VEGF expression in chondrocytes is induced by high-intensity stress and acts in cartilage as an autocrine inducer of matrix metalloproteinases (MMPs). Furthermore, Forsythe et al. (1996) reported that VEGF induction in chondrocytes by mechanical overload is linked to activation of the hypoxia-induced transcription factor-1 (HIF-1), which is known to bind to hypoxia response element (HRE) in the human VEGF gene promoter. Pufe et al. (2004) also reported that after mechanical overload chondrocytes were strongly immunopositive for HIF-1, resulting in induction of VEGF. Consequently, mechanical overload induces HIF-1, and the subsequently generated VEGF activates the chondrocytes autocrinally for producing MMP-1, -3, and -13. Tissue inhibitors of metalloproteinase (TIMP-1 and -2) are then reduced by mechanical overload (Pufe et al. 2004). These findings indicate that VEGF is probably induced in chondrocytes by mechanical overload, probably facilitating hypoxia to mediate the destructive processes associated with OA as an autocrine factor. It must be noted that the presence of hypoxia and its association with VEGF was not the subject of the present study.

Furthermore, we observed that the number of blood vessels and osteoclasts obviously increased in the area subjacent to the hypertrophic cell layer where a number of VEGF-expressing chondrocytes could be detected after a forced jaw opening. It is reported that VEGF played an important role not only in endothelial cell recruitment but also in osteoclast recruitment (Niida et al. 1999; Engsig et al. 2000). Niida et al. (1999) demonstrated that M-CSF and VEGF have overlapping functions in the support of osteoclastic bone resorption. These findings suggested that VEGF produced by chondrocytes might be responsible for migration into cartilage, differentiation, and stimulation of preosteoclasts and osteoclasts. Then, the increase of osteoclasts stimulated by VEGF may induce destruction of cartilage and make vascular invasion into the condylar cartilage easier.

Chondrocyte maturation is considered to be arrested in normal, healthy, articular cartilage whereas at the onset of OA, this arrest may be discontinued. Wong et al. (2003) examined the effect of cyclic tension and cyclic hydrostatic pressure on the expression of VEGF, MMP-13, and TIMP-1 in cultured chondrocytes and showed that VEGF was significantly upregulated by both cyclic tension and hydrostatic pressure. Cyclic hydrostatic pressure downregulated the expression of MMP-13 and upregulated expression of TIMP-1 while cyclic tension upregulated MMP-13 and downregulated TIMP-1. This implies that chondrocyte differentiation is slowed by cyclic hydrostatic pressure and accelerated by cyclic tension. Ohashi et al. (2002) investigated the effects of different magnitudes of dynamic loading on growth plate biology and showed that cartilage mineralization was suppressed and VEGF expression in chondrocytes was accelerated proportionally with the magnitude of applied force. Considering these findings, chondrocytes, especially hypertrophic chondrocytes, appear to have evolved complex mechanoresponsive mechanisms.

In the present study, we found that in both the experimental groups, VEGF was detected in more than 80% of the chondrocytes of the mature and hypertrophic cell layer. The 10-day group showed an increase in the number of cartilage cells without marked pathological changes in the TMJ while the 20-day group showed obvious pathological changes of the articular cartilage and disc with a decrease in number of cartilage cells and a percentual increase of VEGF immunoreactive cells. Judging from our results, chondrocyte maturation also depends on the rate and period of mechanical overloading.

In conclusion, VEGF was expressed by articular chondrocytes (especially in the hypertrophic cell layer) in the mandibular condyle after mechanical overloads by a forced mouth opening. The percentage of VEGF immunopositive chondrocytes increased significantly with the period of forced mouth opening. It is suggested that VEGF plays an important autocrine or paracrine role in the initiation and progression of OA in the TMJ.

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*Original Article*

# Platelet-derived Growth Factor Enhances Proliferation and Matrix Synthesis of Temporomandibular Joint Disc–derived Cells

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**Abstract:** Platelet-derived growth factor (PDGF) is an essential signaling molecule for wound healing and tissue repair. This study was aimed at evaluating the effect of PDGF on the proliferation of temporomandibular joint (TMJ) disc–derived cells and extracellular matrix synthesis. The number of cultured cells were counted by COULTER Z1. The assay for collagen synthesis was performed using a sircol soluble collagen assay. Hyaluronic acid (HA) synthesis was analyzed by a high performance liquid chromatography. The expression of collagens, matrix metalloproteinases (MMPs), and the tissue inhibitors of metalloproteinases (TIMPs) were examined using SYBR Green in terms of the RNA levels. PDGF treatment significantly ($P < .01$) increased the proliferation rate of the disc-derived cells as compared with the controls when the dose was 5 ng/mL or greater. Treatment with more than 5 ng/mL PDGF resulted in an amount of collagen synthesis significantly ($P < .01$) higher than the controls. HA synthesis was maximal with 5 ng/mL PDGF treatment. Quantitative real-time polymerase chain reaction analyses showed that treatment with 5 ng/mL of PDGF-BB upregulated the mitochondrial RNA levels of type I and II collagens, MMPs, and TIMPs within 6 hours. It is concluded that PDGF, if its concentration is optimal, enhanced proliferation and matrix synthesis of TMJ disc–derived cells, indicating that PDGF may be effective for use in tissue engineering of the TMJ disc. (*Angle Orthod* 2006;76:486–492.)

**Key Words:** Temporomandibular joint disc; Tissue engineering; Platelet-derived growth factor

## INTRODUCTION

The temporomandibular joint (TMJ) disc is located between the articulating surfaces of the mandibular condyle and the glenoid fossa and facilitates condylar movement.[1,2] The disc is composed of variable amounts of cells and the supporting extracellular matrices containing large amount of type I collagen, together with type II and III collagens, and proteoglycans.[3–5] During turnover, development, and degeneration of the TMJ disc matrix, the disc cells produce a wide range of matrix metalloproteinases (MMPs), which degrade the components of the extracellular matrix.[6] The expression and activity of MMPs are highly regulated at the level of transcription by growth factors, cytokines, and the corresponding tissue inhibitors

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of metalloproteinases (TIMPs). The TIMPs are very important regulatory factors in the activity of MMPs and tissue destruction and in a disease onset, often correlate with an imbalance between MMPs and TIMPs.[7]

The TMJ discs in patients with severe osteoarthritis (OA) generally exhibit degenerative changes such as hyalinization and collagenization and structural changes such as perforation and thinning.[8,9] Furthermore, the running patterns of collagen fibers or bundles also become more irregular in a damaged disc than in the normal one.[9] Although tissue engineering of the TMJ disc is an essential necessity for actual TMJ reconstruction, only a few studies are found in the literature, providing little available information for the effective remedy of TMJ-OA.[10,11]

Platelet-derived growth factor (PDGF) is a well-known signaling molecule, which promotes the proliferation of fibroblasts.[12] PDGF is released during tissue regeneration from numerous cells such as fibroblasts, chondrocytes, and glia cells.[13–15] In addition, it has powerful stimulating effects on the proliferation of these cells and in promoting the synthesis of collagens and the related chemical mediators.[12,14]

PDGF exists as a form of homodimers or heterodimers of PDGF A and B chains. PDGF-AA effectively binds to only the $\alpha$-subunit of PDGF receptor, whereas PDGF-BB binds to both $\alpha$- and $\beta$-subunits.[16] Although both PDGF-AA and -BB are strong mitogens, only PDGF-BB induces phenotypic transformation of fibroblast cells.[17]

Considering the composition of TMJ disc cells and the extracellular matrices, PDGF-BB may be effective for TMJ disc cell proliferation and differentiation. The aim of this study was to evaluate the effect of PDGF-BB on cell proliferation and the synthesis of extracellular matrices in simultaneously cultured TMJ disc–derived cells. Furthermore, we examined whether or not regulation of MMPs and TIMPs in cultured disc-derived cells might be mediated by PDGF-BB.

## MATERIALS AND METHODS

### Cell isolation and the culture

Five 7-week-old, Wistar strain male rats were euthanized with overdosed pentobarbital according to the guidelines of the Animal Care and Use Committee at Hiroshima University. After the sacrifice of animals, the TMJ discs were dissected out under sterile conditions. The discs were immediately dipped together for 1–2 seconds in 95% ethyl alcohol and rinsed for 2 minutes in sterile phosphate-buffered saline with Fungizon (GIBCO BRL, Paisley, UK). The discs were minced and digested in 10 mL of Dulbecco's modified Eagle's medium (DMEM; NISSUI, Tokyo, Japan) with 0.1% trypsin (Difco, Detroit, Mich) and 0.15% collagenase (Wako, Osaka, Japan) at 37° for 1 hour. Disc cells isolated by the enzymatic digestion were cultured simultaneously in DMEM containing 10% fetal bovine serum (FBS; Mitsubishikasei, Tokyo, Japan). The disc-derived cells of three or five passages were used for the following analyses.

### PDGF stimulation

The cultured disc-derived cells were seeded at a cell density of $4 \times 10^3$/well into 24-well plates. One day later, the number of cells was counted by COULTER Z1 (Coulter Electronics, Herpendon, UK) and defined as a base on zero day. At zero day, PDGF-BB (Santa Cruz, Calif) was added to DMEM with 5% FBS at final concentrations of 0.5, one, five, 10, 20, 40, and 80 ng/mL. Cells without PDGF-BB served as the control. Three days later, the culture medium containing PDGF-BB was retrieved and replaced with the same volume of fresh medium containing PDGF-BB. The cells were counted similarly using COULTER Z1 on days 3 and 6.

### Analysis of collagen synthesis

The disc-derived cells were seeded into six-well plates. PDGF-BB was added to DMEM at the final concentrations of 0, 1, 2, 5, 10, 20, and 30 ng/mL. The cell-conditioned medium collected on the third day as described above was used for the analysis of collagen synthesis. After centrifugation at 12,000 rpm, the supernatants were collected. Then, the assay for collagen synthesis was performed using Sircol™ Soluble Collagen Assay (Biocolor, Belfast, Northern Ireland). After centrifugation, each supernatant of 100 $\mu$L was assayed. One milliliter of Sircol dye reagent, which specifically binds to collagen, was added to each sample and mixed for 30 minutes at room temperature. After centrifugation at 12,000 rpm, the pellet was suspended in 1 mL alkali reagent (0.5 M NaOH) included in the kit, and the optical density was evaluated at a wavelength of 540 nm with a spectrophotometer. The values in the test samples were compared with the values obtained with collagen standard solutions provided by the manufacturer, which were used to construct a standard curve.

### Analysis of hyaluronic acid synthesis

The cells were seeded into six-well plates. PDGF-BB was added to DMEM at the final concentrations of 0, 1, 2, 5, 10, 20, and 30 ng/mL. The cell-conditioned medium collected on the sixth day as described above was used for the analysis of hyaluronic acid (HA) synthesis. Cells were removed from the medium by cen-

trifugation at 1500 × *g* for 15 minutes at room temperature. The supernatants were diluted 10-fold with distilled water before digestion with hyaluronidase (HAase) (Seikagaku Corp, Tokyo, Japan). The mixture was ultrafiltered after digestion with HAase, and the filtrate was analyzed by a high performance liquid chromatography (HPLC). Elution was carried out with seven mM Tris, (pH 7.4) 200 mM NaCl at a flow rate of 0.3 mL/min. The apparent molecular mass of HA was estimated using the molecular weight markers. During the HPLC gel filtration, absorbance was monitored at a wavelength of 206 or 232 nm with ultraviolet/visible and refractive index monitors.

### RNA isolation and analysis

Total RNA was extracted from the cultured cells treated with and without five ng/mL PDGF-BB, using an RNeasy® Mini kit (QIAGEN, Tokyo, Japan). The complementary DNA (cDNA) was produced from the extracted total RNA of 0.5–1.0 μg using an Omniscript™ Reverse Transcriptase kit (QIAGEN). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR master mix. First-strand cDNA obtained by reverse transcription of total RNA was amplified by PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems Japan, Tokyo, Japan), and the fluorescence was collected thrice during each cycle as described below. PCR was performed at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. All the samples were run in triplicates, and the readings were normalized using the SYBR Green Master mix (QIAGEN). Glyceroaldehyde-3-Phosphate dehydrogehase (G3PDH) was used as an internal control in each run. Normalized fluorescence was plotted against cycle number (amplification plot), and the threshold suggested by the software was used to calculate $C_t$ (cycle at threshold). Results of the real-time PCR were expressed as $C_t$, and the expression levels of collagens, MMPs, and TIMPs were indicated by the number of cycles required to achieve the threshold level of amplification. The primers used in this study were summarized in Table 1.

### Statistical analysis

Results were expressed as the mean ± standard deviation (SD), and statistical comparisons of the means were performed using multivariate analysis of variance (ANOVA) at $P < .05$ level. The variances are almost the same or insignificant when validated by *F*-test, an independent Scheffe's test was performed as a post hoc test at 5% level of significance.

**TABLE 1.** List of Primers Used for Quantitative Real-time PCR[a]

| Gene | Primer Sequences 5′→3′ |
|---|---|
| G3PDH | Forward: ACCACAGTCCATGCCATCAC |
| | Reverse: TCCACCACCCTGTTGCTGTA |
| Type I collagen | Forward: CTGCCTGCTTCGTGTAACT |
| | Reverse: CCCTCTGTTAAAGTGTACCTGA |
| Type II collagen | Forward: CTCACGCCTTCCCATTGT |
| | Reverse: TCCTAGAGTGACTGCGGT |
| MMP-3 | Forward: GGATCTTCACAGTTGGAGTT |
| | Reverse: GCACATGCTAGAGTAAGGAA |
| MMP-8 | Forward: TGGGCTCTAAGTGCCTATGA |
| | Reverse: TGTCGTATCTCCAGCATTG |
| MMP-13 | Forward: GGCAGACATAGTAAGTAGA |
| | Reverse: TAAGCACCAAGTGTCAG |
| TIMP-1 | Forward: TCCTGGTTCCCTGGCATAAT |
| | Reverse: GGCAAAGTGATCGCTCTGGT |
| TIMP-2 | Forward: GTGACTTTATTGTGCCCTGG |
| | Reverse: GCCCATTGATGCTCTTCTCT |

[a] MMP indicates matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinases.

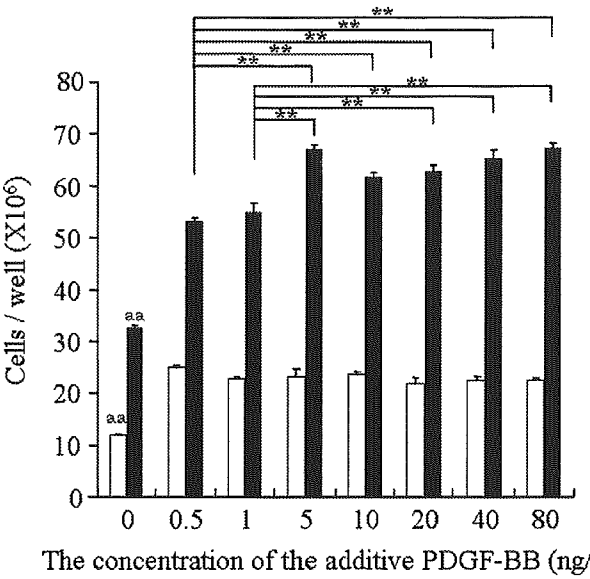


**FIGURE 1.** Effects of PDGF-BB on the proliferation of the TMJ disc–derived cell culture. □ third day ■ sixth day aa: $P < .01$ compared with the values in the PDGF treatment groups at the same day. ** Significance of difference between the values ($P < .01$) as tested with Scheffe's test. PDGF indicates platelet-derived growth factor; TMJ, temporomandibular joint.

## RESULTS

### Effects of PDGF-BB on cell proliferation

On the third day, PDGF-BB treatment significantly ($P < .01$) increased proliferation of the disc-derived cells irrespective of its concentration, and the proliferation rate was more than two times that of the controls (Figure 1). On the sixth day, 5 ng/mL PDGF-BB treatments also increased cell numbers by more than double the controls. The effect was maximal at a concentration of 5 ng/mL (Figure 1). Irrespective of the

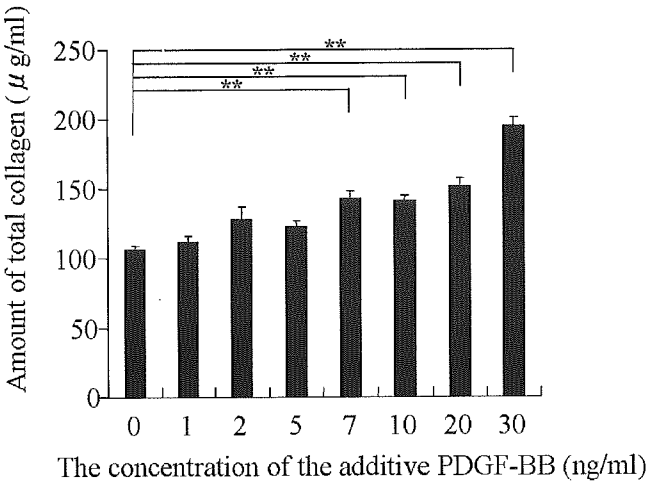


**FIGURE 2.** Effects of PDGF-BB on the collagen synthesis. ** Significance of difference between the values ($P < .01$) as tested with Scheffe's test. PDGF indicates Platelet-derived growth factor.

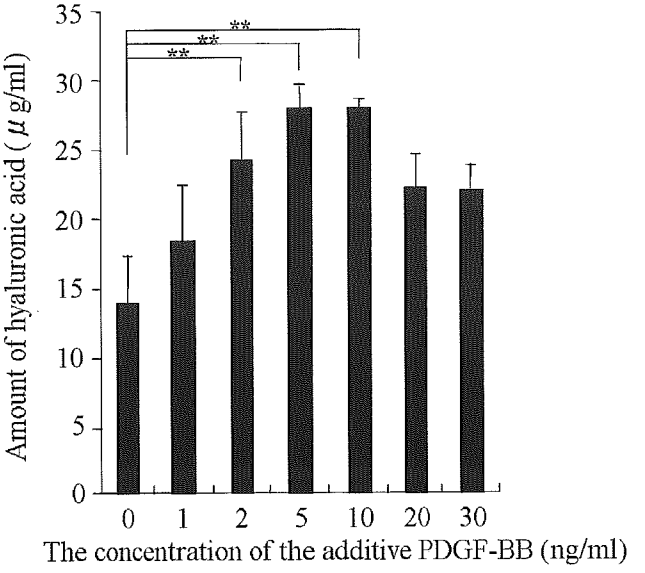


**FIGURE 3.** Effects of PDGF-BB on the hyaluronic acid synthesis. ** Significance of difference between the values ($P < .01$) as tested with Scheffe's test, PDGF indicates platelet-derived growth factor.

concentration of additive PDGF-BB and the culture period, the proliferating ability was significantly ($P < .01$) enhanced by the addition of PDGF-BB as compared with the controls.

### Collagen synthesis

The synthetic activity for collagen was stimulated by PDGF-BB. As the concentration of additive PDGF-BB increased, the amount of synthetic collagen increased by 126–162% of the control. Collagen synthesis was maximal with 30 ng/mL PDGF-BB treatment. By the addition of more than 5 ng/mL PDGF-BB, the amount of synthetic collagen was significantly ($P < .01$) increased when compared with the controls (Figure 2).

### HA synthesis

Treatment with PDGF-BB of 2, 5, and 10 ng/mL produced a significant ($P < .01$) increase in the synthesis of HA as compared with the controls (Figure 3). When the concentration was greater than 10 ng/mL, HA synthesis was decreased substantially.

### Mitochondrial RNA expression of collagens, MMPs, and TIMPs

Quantitative real-time PCR analyses were performed for the expression of type I and II collagens mitochondrial RNAs (mRNAs) isolated from the cultured cells treated with and without PDGF-BB for 0, 1, 3, or 6 hours. Treatment with PDGF-BB significantly ($P < .01$) increased expression levels of type I collagen at 1 hour and type II collagen at 6 hours (Figure 4). In contrast, the expression levels of both collagens did not vary significantly in the control.

PDGF-BB treatment increased transcription levels of MMP-3 at 6, 12, and 24 hours, MMP-13 at 6 and 12 hours, and TIMP-1 at 6, 12, and 24 hours in the cells (Figure 5). Both MMP-3 and MMP-13 activities were detected mostly in the same periods, whereas MMP-8 could not be detected in both groups, indicating that PDGF-BB induced no expression of MMP-8.

## DISCUSSION

The TMJ disc–derived cells used in this study resembled fibrocytes, fibroblasts, and fibrochondrocytes, but it would not be appropriate to regard these cells as chondrocytes.[18] Landesberg et al[18] reported that the TMJ disc–derived cells have characteristics of both fibroblasts and chondrocytes.

Only one study on the tissue engineering of the TMJ disc has been reported recently. In this study, the effect of growth factors including PDGF on the disc cell was examined in terms of cell proliferation and matrix synthesis in the cell culture.[10] They demonstrated that PDGF exerted a moderate, but not powerful effect on disc regeneration. They used PDGF-AB but not PDGF-BB as a promoting factor of disc cell metabolism. PDGF has three types of receptor; αα-receptor homodimers, αβ-receptor heterodimers and ββ-receptor homodimers. Although PDGF-BB can bind to all receptors, PDGF-AB cannot bind to ββ-receptor.[19] Furthermore, expression of β-receptor has been mainly observed in epithelial cells after cutaneous injury and inflammation.[20,21] From these findings, it would be assumed that PDGF-BB is the most beneficial for triggering a marked increase of cell proliferation and syn-

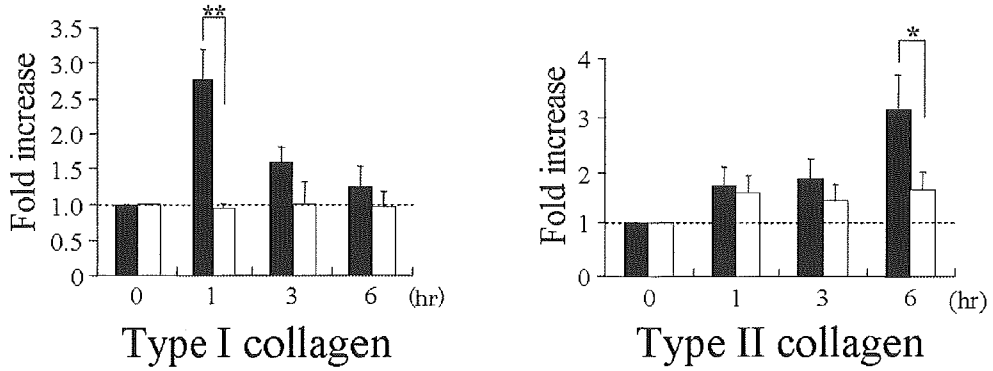


**FIGURE 4.** Expression of type I collagen and type II collagen mRNA ■ PDGF □ Control * Significance of difference between the values ($P < .05$) as tested with Scheffe's test. ** Significance of difference between the values ($P < .01$) as tested with Scheffe's test. PDGF indicates platelet-derived growth factor; mRNA, mitochondrial RNA.

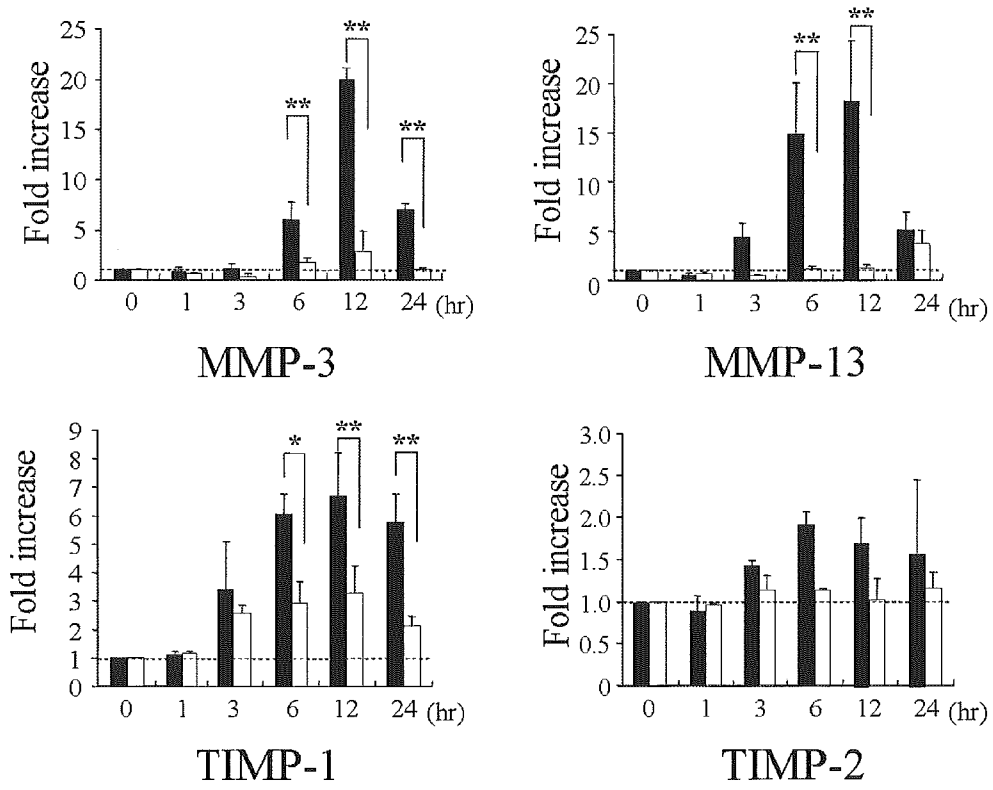


**FIGURE 5.** Expression of MMPs and TIMPs mRNA ■ PDGF □ Control * Significance of difference between the values ($P < .05$) as tested with Scheffe's test. ** Significance of difference between the values ($P < .01$) as tested with Scheffe's test. PDGF indicates platelet-derived growth factor; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; and mRNA, mitochondrial RNA.

thesis of the extracellular matrix, compared with PDGF-AA and -AB.

In this study, PDGF-BB stimulated cell proliferation by 200% at maximum. Furthermore, disc-derived cells responded to PDGF-BB dose dependently and reached its maximum effect at a concentration of 5 ng/mL. The cells of the TMJ disc resemble fibrocytes, fibroblasts, and fibrochondrocytes, but it would not be appropriate to refer to these cells as chondrocytes.[18] Because the number of cells present in the disc was very small,[18] it indicates that enhancement of cell proliferation induced by PDGF-BB is of great usefulness for the repair of the TMJ disc.

Although all the joint tissues have various amounts and types of collagens with an important function for the mechanical resilience of the tissues under mechanical stress,[22] the changes in the matrix content and the modifications of the molecular structure within the disc affect the TMJ function. In fact, it is important to know various collagen components of the disc be-

cause this is indispensable for understanding the molecular processes in disc degeneration and the need in disc repair. Thus, a newly formed collagenous matrix not only requires the correct molecular constituents but also an appropriate supramolecular arrangement of them for proper tissue functioning. In this study, upregulation of MMP-3 (stromelysin 1) and -13 (collagenase 3) was detected in cultured cells with PDGF-BB stimulation at 6 and 12 hours. Meanwhile, the mRNA levels of TIMPs were also upregulated simultaneously, and interestingly, upregulation of TIMPs occurred before that of MMPs.

The functional integrity of connective tissue depends primarily on that of its extracellular matrix and its balanced turnover. Thus, a limited degradation of matrix components (catabolism) is important as much as a sufficient synthesis of them (anabolism).[22] The activities of MMPs and TIMPs and the ratio between them are well understood as essential for physiological remodeling of the disc. In contrast, tissue destruction in the course of the onset of diseases often correlates with an imbalance of MMPs over TIMPs.[23] Therefore, the time-dependent upregulations of these enzymes induced by the administration of PDGF-BB in this study imply appropriate events for TMJ disc tissue engineering.

HA is an important component of the TMJ disc and synovial fluid. HA has a viscoelastic character, which provides joint lubricating ability and enables the condyle and disc to move harmoniously during function. Thus far, HA synthesis in the disc cells is, presumably, of a great importance for TMJ lubrication. Furthermore, it has been reported that HA synthesis is related to cell proliferation,[24] indicating that synthetic HA will also modulate disc cell proliferation.

Our results showed an increase of HA synthesis from the disc cells after stimulation by PDGF-BB. In this in vitro study, although optimal concentrations of PDGF-BB for tissue engineering of the TMJ disc were not determined, it is anticipated that the use of proper concentrations may also lead to enhancement of cell proliferation and matrix synthesis in vivo.

In conclusion, it is shown that PDGF-BB, if its concentration is optimal, enhances proliferation and matrix synthesis of TMJ disc–derived cells. It is also emphasized that PDGF-BB may have future therapeutic potential for the treatment of damaged TMJ discs resulting from various degenerative diseases such as TMJ-OA.

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