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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.

KEY WORDS: Temporomandibular joint disc, Tissue engineering, Platelet-derived growth factor.

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INTRODUCTION Return to TOC

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 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 α-subunit of PDGF receptor, whereas PDGF-BB binds to both α- and β-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 Return to TOC

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 4×10^3/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 μL 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 centrifugation 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.

RESULTS Return to TOC

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 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 Return to TOC

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 synthesis 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 because 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.

ACKNOWLEDGMENTS

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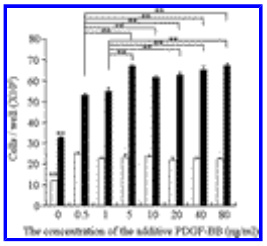
TABLE 1. List of Primers Used for Quantitative Real-time PCR[a]

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

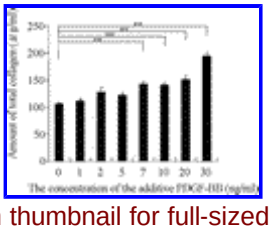
[a] MMP indicates matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinases.

FIGURES Return to TOC



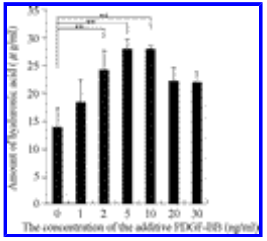
Click on thumbnail for full-sized image.

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



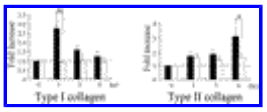
Click on thumbnail for full-sized image.

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



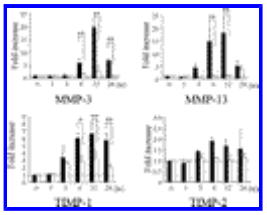
Click on thumbnail for full-sized image.

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



Click on thumbnail for full-sized image.

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



Click on thumbnail for full-sized image.

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

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