Induced Differentiation of Rat Periodontal Ligament-Derived Cells Using Growth Factor Cocktails

Abstract

Objective: Establishing effective methods for inducing osteoblasts from stem cells is important for developing guided bone regeneration (GBR) therapy. We investigated a method of osteogenic cell induction by combining various growth factors (GFs) to safely, efficiently, and in large quantities induce stem cells to differentiate into dental tissue-forming cells such as osteoblasts in vitro and to induce regeneration of dental tissues as quickly as possible by returning these differentiated cells to the body.

Materials and Methods: We aseptically removed molar periodontal ligaments of Wistar rats (4-week–old males; n = 40) and isolated and cultured the cells. We then determined GFs with high expression of GF receptors in cells using quantitative real-time polymerase chain reaction (PCR); these GFs were combined to a culture medium containing 100 nM dexamethasone (Dex). Cell counts were measured, and cell differentiation was evaluated using alkaline phosphatase staining after 7, 14, 17, and 21 days of culturing. Expression of transcription factors such as runt-related transcription factor 2 (Runx2) and Osterix was also examined using immunohistochemical staining.

Results: Quantitative real-time PCR revealed the following four types of GFs with high expression of GF receptors in cells: bone morphogenetic protein (BMP)-2, basic fibroblast growth factor (bFGF), insulin, and insulin-like growth factor (IGF)-1. A differentiation induction experiment using these GFs showed no induced differentiation even after 21 days of culturing in a culture medium containing bFGF. In contrast, when insulin, IGF-1, and Dex were added to a culture medium containing BMP-2, a remarkable number of Runx2-immuno-reactive (IR) cells were observed from day 14, with Osterix-IR cells noted from day 17 of induced differentiation.

Conclusion: BMP-2, insulin, IGF-1, and Dex promote induced differentiation of osteogenic cells, whereas bFGF promotes the proliferation of cells.

Keywords: Mesenchymal Stem Cell (MSC), Differentiation, Growth Factor (GF)

Introduction

According to the Survey of Dental Diseases by the Japanese Ministry of Health, Labour and Welfare in 2016, people aged ≥75 years have on an average at least 10 teeth missing. Prosthetic treatment remains vital for improving chewing and eating-related quality of life (QOL) and activities of daily living (ADL) for these people.

One option of prosthetic treatment is oral implants, which cause less discomfort than dentures, allow for chewing conditions similar to those with natural teeth if osseointegration is successfully achieved, and lead to improved QOL and ADL. Osteoblast proliferation and differentiation are involved in achieving osseointegration, which is the key to successful oral implantation, and thus must be achieved as quickly and safely as possible.

Although osteoblasts are differentiated from Mesenchymal Stem Cells (MSCs), the body contains only a small number of MSCs. Therefore, differentiating all MSCs into osteoblasts using existing induction methods is essential. Investigation of effective methods for inducing differentiation of MSCs into osteoblasts and application of these methods for Guided Bone Regeneration (GBR) therapy are underway.

To understand the induction of MSC differentiation into osteoblasts, the introduction of growth factors (GFs) should be examined. Numerous factors, including bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), stromal...
cell-derived factor-1, insulin-like growth factor (IGF)-1, and runt-related transcription factor 2 (Runx2), play the role of facilitators or modulators of induced differentiation of MSCs into osteoblasts [1]. GFs such as BMP-2, BMP-7, basic fibroblast growth factor (bFGF) [4,5], platelet-derived growth factor [6], and IGF-1, have clinical applications and induce the differentiation of undifferentiated cells, found in periodontal ligament (PDL)-derived cells, into osteoblasts, cementoblasts, and other cells [7,8]. Of these GFs, BMP-2, a protein that belongs to the TGF-β superfamily, controls processes such as cell differentiation, proliferation, and death [9]. Furthermore, under serum-free α-minimal essential medium (MEM) conditions, the addition of BMP to human PDL-derived cells increases the activity of alkaline phosphatase (ALP), which is an index of cell differentiation activity [10]. Moreover, an experiment involving an osteogenesis model created using three-dimensional culturing of rat bone marrow-derived cells reported that BMP-2, IGF-1, TGF-β1, vascular endothelial GF, ALP, and osteocalcin to be factors were necessary for osteogenesis and that various GFs form complexes to mutually communicate and contribute to bone formation [11].

In this study, we report an experiment in which we efficiently induced osteogenic cells using cocktail culture medium containing different combinations of multiple GFs to effectively promote MSCs to differentiate into osteoblasts in vitro and to as quickly as possible return these differentiated cells to the body to achieve osseointegration in oral implantation treatment.

Materials and Methods

This experiment was conducted in accordance with approval from the Animal Research Committee of Osaka Dental University (approval number: 14-01009).

Cells and culturing methods

Molars of Wistar rats (4-week-old males; n = 40) were extracted and incubated in 10 mL of PBS containing 20 mL/mL of kanamycin (Meiji Seika Pharma, Tokyo, Japan) for 2 h at 4°C. Next, the molars were incubated in 10 mL of Eagle’s MEM (Sigma Aldrich, MO, USA) containing 10% fetal bovine serum (Biological Industries Ltd., Israel), antibiotic–antimycotic solution (100×; Gibco Laboratories, NY, USA), L-ascorbic acid (50 μg/mL; Sigma Aldrich), and 2 mM L-glutamine (Gibco Laboratories) were added to Eagle’s MEM to create a base culture medium. The cells were seeded and cultured in a 100-mm dish (Asahi Techno Glass Co., Ltd., Shizuoka, Japan) that contained the base culture medium. MSCs obtained from rat molar PDLS (hereafter PDLMSCs) were used in the experiments.

Identifying a GF cocktail that efficiently induces osteo-
blast differentiation

Candidate GFs that efficiently and effectively induce differentiation of PDLMSCs into osteoblasts were factors with clinical applications in the current medical field. These GFs were combined to form different cocktails that were added to the base culture medium to induce differentiation. In addition, mRNA expressions of each GF and insulin receptor were analyzed using quantitative real-time polymerase chain reaction (PCR), and receptor agonists that were strongly expressed were considered to be candidates for creating the cocktail.

Total RNA was extracted from PDLMSCs using the RNeasy plus Micro Kit (QIAGEN, Tokyo, Japan). RNA was then subjected to real-time PCR analysis using the One Step SYBR Prime Script PLUS RT-PCR Kit (Takara Bio Inc., Shiga, Japan) and Opticon Real-Time PCR System (MJ Research, MA, USA) according to the manufacturer’s instructions. The candidate GFs, receptor primers of each GF, and quantitative real-time PCR conditions are shown in Table 1.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Receptor</th>
<th>Left Primer</th>
<th>Right Primer</th>
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<td>IGF1</td>
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<td>17β-estradiol</td>
<td>ERα</td>
<td>cagactgactgactgactctg</td>
<td>ccgctgacatctctctca</td>
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Optimal factors that induced the differentiation of PDLMSCs into osteoblasts were evaluated by comparing receptors of each factor expressed in PDLMSCs that were cultured under the following conditions:

- Condition (a): PDL cells at 12 h after harvesting (12 h of adhesion to obtain only adherent cells in PDL), start of culturing (0 h)
- Condition (b): Culturing with Dex added to Eagle’s MEM (24 h)
- Condition (c): Culturing using Eagle’s MEM (24 h)

Conditions (a)–(c) were tested by t-test, with a significance level of 5%. GFs that exhibited a significant difference were considered to be candidates for the GF cocktail to be used in this study.

Examination of optimal GF concentrations and optimal combination of GFs

GFs, exhibiting particularly high levels of expression based on the quantity of GF receptor mRNAs whose expression were confirmed in Section 2, were examined. BMP-2 and Dex concentrations were assessed based on the previous studies [3,12,13]. BMP-2 and Dex concentrations (100 ng/ml and 100 nM, respectively) and equivalent amounts, i.e., 2-, 3-, 5-, 0.5-, 0.2-, and 0.1-fold amounts, were used. The following five groups were created for each cocktail to test the induction of osteoblast differentiation, and the groups were compared with each other. An experiment using human MSCs revealed that a cocktail containing BMP-2 and Dex calcified the cells without increasing adipogenesis–associated markers and that Dex was necessary for calcification [12]. Therefore, BMP-2 and Dex were added to the cocktail culture media of groups other than Group A.

- Group A (control): base culture medium
- Group B (BDinsulin): BMP-2 (100 ng/ml), Dex (100 nM), and insulin (50 µg/ml)
- Group C (BDI): BMP-2 (100 ng/ml), Dex (100 nM), and IGF-1 (100 ng/ml)
- Group D (BDII): BMP-2 (100 ng/ml), Dex (100 nM), insulin (50 µg/ml), and IGF-1 (100 ng/ml)
- Group E (BDIF): BMP-2 (100 ng/ml), Dex (100 nM), IGF-1 (100 ng/ml), and bFGF (50 ng/ml)

PDLMSCs that reached 70% confluence were used in each group and were seeded into a 48-well plate (AGC Techno Glass Co., Ltd.) at a cell concentration of 1.0 × 10^4 cells/ml. Cell counts were measured after 7, 14, and 21 days of culturing to evaluate cell proliferation (0.5% trypan blue stain solution; Nacalai Tesque, Japan). Staining of ALP (Alkaline Phosphatase Substrate Kit, BIORAD, CA, USA), an osteoblast marker enzyme, was also performed to compare the state of osteoblast differentiation in PDLMSCs.

Examination of marker expression of osteoblast differentiation in osteoblast-induced cells

To examine whether cells differentiated into osteoblasts, the enzyme-labeled antibody method was used to investigate the expression of the transcription factors Runx2, which is essential for differentiation of MSCs into osteoblasts, and Osterix, which is expressed downstream of Runx2 and is involved in the differentiation of preosteoblasts into immature osteoblasts [14]. The primary antibodies used for staining with this method are as shown below.

- Rabbit anti-Runx2 (1:1000, 1 h, room temperature (RT); Abcam, UK)
- Rabbit anti-Osterix (1:500, 1 h, RT; Abcam)

The following secondary antibodies were used.

- Biotinylated goat anti-rabbit IgG (1:400, 30 min, RT; Vector Laboratories, USA)
- Avidin-biotin-peroxidase complex (30 min, RT, Elite ABC kit; Vector Laboratories).

Distilled water containing 0.05 % 3,3′-diaminobenzidine (2 min, RT, Vector Laboratories), 0.2 % nickel sulfate, and 0.01 % hydrogen peroxide.

Analysis

PDLMSCs cultured in a 48-well plate were photographed using a phase-contrast microscope [Olympus CKX41, Olympus; Olympus FX380 3CCD digital camera system (OS: Windows 7; Microsoft, CA, USA) connected with the FLvFs software (Flovel Image Filling System, Tokyo, Japan)]. Cell counts were statistically analyzed using one-way analysis of variance (Excel 2010; Microsoft, USA, statistically significant: * p < 0.05).

Cells stained using the enzyme-labeled antibody method were observed using an optical microscope (Olympus BX41 light microscope, Olympus) and photographed (Olympus FX380 3CCD digital camera system, connected with a FLvFs software [Flovel Image Filling System]).

Results

Expression of various GFs and GF analog receptor mRNAs

When PDLMSCs were cultured for 24 h, relative mRNA expression levels of BMP-2 receptors, BMPR-Ib and BMPR-II; the bFGF receptor, FGFR2; and the GF receptors, insulin R and IGF-1R, were all high (Figure 1). Furthermore, relative mRNA expression levels were higher in the culture medium condition (b) containing Dex and IGF-1R than in the condition (c) (p<0.05, Figure 1).

Therefore, a cocktail containing BMP-2, bFGF and IGF-1 along Dex was created and subjected to an experiment in which PDLMSC differentiation into osteoblasts was induced. Insulin, an analog of IGF-1, was also included as a candidate GF because its
the cells were viable until the end of the experiment, but no signs of marked differentiation were observed during the experiment itself at amounts equivalent to or lower than 0.2 fold. In addition, when a 2-fold amount of BMP-2 was added, no marked difference in differentiation was observed compared with when 100 μM BMP-2 was administered. Furthermore, differentiation was not achieved during the experiment in the culture medium not containing BMP-2. Therefore, regarding the effects on the body, an experiment in which BMP-2 (100 μM) and Dex (100 nM) were added to the differentiation-inducing culture medium was conducted.

Receptor mRNA was markedly expressed.

**Optimum concentrations of BMP-2 (data not shown)**

PDLMSCs showed more pronounced ALP-stained images when cultured in a combination of BMP-2 (100 μM) and Dex (100 nM) than when cultured with BMP-2 (100 ng / mL) and Dex (100 nM) alone. These pronounced stained images were observed from day 14 of culturing.

In the culture medium containing a combination of BMP-2 and Dex at concentrations greater than 300 μM and 300 nM, respectively, PDLMSCs died within 7 days of induced differentiation. In contrast, in an experiment where BMP-2 was added to the culture medium in 0.5-, 0.2-, and 0.1-fold amounts, the cells were viable until the end of the experiment, but no signs of marked differentiation were observed during the experiment itself at amounts equivalent to or lower than 0.2 fold. In addition, when a 2-fold amount of BMP-2 was added, no marked difference in differentiation was observed compared with when 100 μM BMP-2 was administered. Furthermore, differentiation was not achieved during the experiment in the culture medium not containing BMP-2. Therefore, regarding the effects on the body, an experiment in which BMP-2 (100 μM) and Dex (100 nM) were added to the differentiation-inducing culture medium was conducted.

**Chronological observation of PDLMSCs using phase-contrast microscopy and ALP staining**

![Figure 1: The expressions of an individual mRNA of each receptor of growth factors (relative amount, *p<0.05).](image)

- (a) Start of culturing (0 h)
- (b) Culturing with Dex added to Eagle’s MEM (24h)
- (c) Culturing using Eagle’s MEM (24h)

![Figure 2: Phase contrast micrographs of PDLMSCs (arrows).](image)
The following four types of GFs were used: BMP-2 (100 μM), insulin (5 μg / mL), IGF-1 (100 ng / mL), and bFGF (50 ng / mL). When observed using a phase-contrast microscope, PDLMSCs increased over time in all groups, from Group A (control) to E (BDIF; p>0.05, Figures 2 and 3). In Group D (BDII), calcified masses began to appear at 7 days after initiating induced differentiation and were noticeable after 17 days. In Groups A (control), B (BDinsulin), and C (BDI), calcified masses appeared at 10 days after initiating induced differentiation. Meanwhile, in Group E (BDIF), no calcified masses appeared even after 21 days of induced differentiation.

ALP-stained images of PDLMSCs at 14 days after initiating induced differentiation revealed marked staining in Group D (BDII) but almost no staining in Group E (BDIF; Figure 4). Therefore, Groups A (control), B (BDinsulin), C (BDI), and D (BDII) were chosen for use in the PDLMSC-induced differentiation experiment.

Expression of osteoblast differentiation markers

Two transcription factors were used as osteoblast differentiation markers: Runx2, which is essential to the differentiation of MSCs into osteoblasts, and Osterix, which is expressed downstream of Runx2 and is involved in the differentiation of preosteoblasts into immature osteoblasts [14]. Expression of these transcription factors was examined to verify the degree of differentiation of PDLMSCs into osteoblasts.

Almost no Runx2-immuno-reactive (IR) cells were observed among PDLMSCs in any of the culture media up to 10 days after initiating induced differentiation. Few Runx2-IR cell masses were observed after 14 days in Group D (BDII) and 17 days in Groups B (BDinsulin) and C (BDI; Figure 5). However, almost no Runx2-IR cell masses were confirmed in Group A (control) even after 21 days of induced differentiation.

Osterix-IR cells were expressed later than Runx2-IR cells. After 17 days of induced differentiation, few Osterix-IR cell
Figure 5: Expressions of Runx2-immuno-reactive (IR) cells (arrows; immunohistochemical staining).

Figure 6: Expressions of Osterix-IR cells (arrows; immunohistochemical staining).
masses were observed in Group D (BDII), and thereafter, a marked number of Osterix-IR cells were observed in cultured PDLMSCs in Groups C (BDI) and D (BDII; Figure 6). However, almost no Osterix-IR cell masses were confirmed in Groups A (control) and B (BDinsulin) even after 21 days of induced differentiation.

**Discussion**

We created a cocktail culture medium containing combinations of different GFs and hormones that were reportedly necessary for induced differentiation of osteoblasts and examined which media efficiently induced differentiation [15-26]. Selected GFs and hormones were essentially those applicable in clinical settings. The initial aim was to examine induced differentiation using a fractional factorial design to efficiently induce osteoblasts [15]. However, after judging that the experiment would proceed more efficiently if the presence or absence of GFs were confirmed, we first performed quantitative real-time PCR to verify the mRNA expressions of GF receptors in PDLMSCs and then refined the candidate GFs and hormones for the cocktails. Of all candidate GFs and hormones assessed for the mRNA expression of GF receptors, BMP-2, BMP-4, bFGF, TGF-β1, and epidermal growth factor were reported to be important in osteogenesis [23]. Using a combination of BMP-9 and TGF-β1 in MSCs promotes differentiation to osteogenesis. Furthermore, the synergistic effect of the BMP-9 and TGF-β1 signal transduction systems promotes osteogenesis and exhibits a dose-dependent synergistic effect on osteogenesis [22,23]. Many GFs are involved in the modulating osteogenesis [12,13], and GF-rich serum can stimulate several biological response processes of cells related to bone regeneration [20]. In human serosal and amniotic membranes, bFGF and TGF-β1 actively regulated bone morphogenetic markers and promoted calcification of the extracellular matrix [24]. These reported findings suggested that creating a cocktail that contained a combination of GFs is an effective method for inducing bone differentiation, and our study results supported these findings.

TGF-β1 was not included in the GF cocktails of this study. The following reports provide the reasoning underlying this decision. BMP, which belongs to the TGF-β superfamily, controls cell processes such as differentiation, proliferation, and death but also induces differentiation of myoblasts into osteoblasts via Smad signals [27]. Meanwhile, TGF-β1 inhibits phosphorylation of Smad1 and Smad5, which are BMP-2- specific Smads, thereby suppressing the action of BMP-2 via a negative feedback action of Smad6 and Smad7 [16]. TGF-β1, detected in PDL and dental pulp, also acts on the differentiation of soft tissues, whereas BMP-2 plays an important role in differentiation to hard tissue cells [16]. Our findings described in Sections 3 and 4 of the Results revealed that calcification occurs after approximately 2 weeks of induced differentiation, even if a GF cocktail containing no TGF-β1 is used. The aforementioned findings suggested that even GF cocktails containing no TGF-β1 are useful as osteogenesis-inducing culture media. Cocktail combinations containing GFs such as BMP-9 [22,23] may also need to be investigated when TGF-β1 is included in the osteogenesis-inducing culture media.

However, one study revealed that adding BMP-2 to osteoblasts strongly down regulated ALP and markedly increased Runx2 and CDH11 [9]. When BMP-2 is added to osteoblasts, ALP and type I collagen levels increase to stimulate cell differentiation; however, this same reported study found that BMP-2 was only sufficient to stimulate cell proliferation and a small amount of differentiation [12].

Nevertheless, BMP-2 has promoted the expression of Runx2, which is essential for osteoblasts differentiation, and Osterix via the transcription factor mash homeobox 2 (Mash2) [14]. In vitro, Osterix affects the differentiation of MSCs into osteoblasts by activating osteogenesis, suggesting that BMP-2 directs the differentiation of MSCs into osteoblasts [14]. Although the role of BMP-2 in osteoblast differentiation is debatable, it is undeniable that BMP-2 controls differentiation, Besides its vital role in the differentiation process.

Section 3 of the Results also showed almost no ALP staining at 14 days after initiating induced differentiation and no calcification even after 21 days of induced differentiation in Group E (BDIF), in which differentiation was induced using a cocktail containing bFGF. Consequently, bFGF was not used in the PDLMSC-induced differentiation experiment. bFGF not only is considered the main stimulus behind cell proliferation [9] but also moderates osteogenesis and expands fatty metamorphosis when human MSCs are used in experiments [12]. Therefore, our results are supported by those of the previous researches. However, the existence of a report that vitamin D3 is an element of calcification only in the presence of bFGF [12] suggests that bFGF could be a useful factor in osteogenesis if its inclusion in the same cocktail as TGF-β1 was considered.

When cultured PDLMSCs were analyzed using quantitative real-time PCR, as described in Section 1 of the Results, the relative expression levels of insulin R and IGF-1R GF receptor mRNA were high. Therefore, these GFs were included as cocktail candidates in our study. Insulin is produced in the pancreas and is a double-stranded peptide that is held together by disulfide bonds. Its higher order structure closely resembles that of IGF because approximately 50% of its amino acids are in the same sequence. Insulin has a lowering effect on blood sugar levels and a metabolic regulatory action inclined toward the assimilation of sugar, protein, and lipid metabolism, but it has a weaker inducing action on cell proliferation and differentiation and inhibitory action on cell death. Insulin is also characterized by a tendency for its blood concentration to instantly increase in response to stimulation and return to a steady state within a short period of time. Meanwhile, IGF is a single-stranded peptide that is held together by disulfide bonds within the molecule. When IGF-1 binds to IGF-IR, the receptor phosphorylates to activate MAP kinase, which suppresses the genes associated with cell survival and growth in the same manner as stem cell differentiation, thereby becoming a strong stimulus underlying cell proliferation and differentiation [9,23,27-30]. Experiments using PDL cells revealed that IGF-1 transduces cell proliferation and differentiation signals via MAP kinase [23,28-30]. Therefore, including insulin and IGF-1 in GF cocktails is considered to be valid. This is also supported by the fact that Group D (BDII), in which a cocktail containing insulin and IGF-1 was used, showed the most calcification and the earliest confirmed expression of Runx2 and Osterix, as described in Sections 3 and 4 of the Results. Meanwhile, the activation of MAP kinase, which transduces the signals of IGF-1, is involved in the induction of the BMP-2 gene in PDL, possibly indicating the need for a more detailed study of the association between MAP kinase, Smad signals, and GFs contained in the cocktails.
developed in our study.

Finally, in Section 4 of the Results, the transcription factors Runx2, which is essential to the differentiation of MSCs into osteoblasts, and Osterix, which is expressed downstream of Runx2 and is involved in the differentiation of preosteoblasts into immature osteoblasts, are examined as markers of osteoblast differentiation [14,25,26]. In MSC lines, Osterix is induced by BMP-2 [14]. Furthermore, the failure of bone and osteoblast formation in the fetal stage in Osterix gene-deficient mice suggests that the Osterix gene plays an important role in bone development and osteoblast formation [14]. BMP-2 promotes the expression of Runx2 and Osterix via Msx2 [14,25,26]. In light of the aforementioned findings, evaluating osteoblast differentiation based on Runx2 and Osterix expressions is essential.

Section 4 of the Results also describes how Osterix expression was observed after Runx2 expression, which similarly indicates that osteoblast differentiation can be evaluated, based on Runx2 and Osterix expressions in terms of the progression of osteogenesis.

In the future, we intend to investigate GFs that were not used in this study to examine more efficient methods of inducing differentiation of MSCs into osteoblasts so as to develop a GBR therapy.

Conclusions

(1) PDL-derived cells differentiate into osteoblasts after approximately 2–3 weeks of induced differentiation.

(2) The results suggested that a cocktail containing BMP-2 and Dex or that containing insulin and IGF-1 in addition to BMP-2 and Dex (four different GFs) is effective when efficiently inducing the differentiation of PDL-derived cells into osteoblasts.

(3) BMP-2, Dex, insulin, and IGF-1 facilitate the induced differentiation of osteogenic cells when inducing the differentiation of PDL-derived cells into osteoblasts. Meanwhile, bFGF facilitates cel proliferation.

Acknowledgment

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References


