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Calcified Nodule Formation *in Vitro* Using Rat Mandibular Incisor Pulp Cells

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ABSTRACT

One great advantage of bone marrow is that a large number of stem cells can be obtained. However, stem cells cannot be taken from the bone marrow of a patient for the purpose of regenerating teeth. In dental practice, it may not be practical to obtain mesenchymal stem cells for the regeneration of the tooth from iliac bone marrow, as in orthopedics. Therefore, establishment of a cell source for tooth regeneration is one of the important problems in the field of regenerative medicine. Although the isolation of undifferentiated mesenchymal stem cells or odontoblasts from dental pulp may be difficult, dental pulp may be a favorable source for stem cells to regenerate a tooth via tissue engineering. As a fundamental study for tooth regeneration, this study was performed in order to clarify the culture periods for proliferation and differentiation using rat dental pulp-derived cells. The results of this study were as follows: Primary culture of the dental pulp cells does not require a long period of time. However, for sufficient proliferation of dental pulp cells to form a calcified nodule in the secondary culture, a long culture period is required. Dexamethasone was essential for the formation of calcified nodules by dental pulp-derived cells. Calcified nodule formation by dental pulp-derived cells *in vitro* required more than one month. The duration of the secondary culture for the dental pulp-derived cells will be much longer.

1. INTRODUCTION

In many of the fundamental experimental studies on osteogenesis, stem cells were obtained from the femoral bone shaft of rats [1-4]. Stem cells may be used in the studies *in vitro* and *in vivo* for tooth regeneration, especially cells from tooth bud or dental pulp tissue. In these *in vivo* or *in vitro* studies for the regeneration of teeth, bone marrow cells were used [5, 6]. However, it is not practical to obtain mesen-

chymal stem cells from iliac bone marrow as in orthopedics [7] for tooth regeneration in dental practice, because the physical stress is severe for the patient. In clinical dentistry in our country, stem cells cannot be taken from the bone marrow of a patient for the purpose of regenerating teeth. Therefore, establishment of a cell source for tooth regeneration is one of the important problems in the field of regenerative medicine. One advantage of using bone marrow cells for tooth regeneration is that a large number of stem cells can be obtained from the femoral bone shaft.

On the other hand, although the isolation of undifferentiated mesenchymal stem cells or odontoblasts from dental pulp may be difficult [8, 9], pulp tissue is considered to be a favorable cellular source for the regeneration of the teeth. Undifferentiated mesenchymal stem cells were reported to exist together with odontoblasts and fibroblasts in dental pulp tissue [10, 11]. Stem cells present in the dental pulp may differentiate autonomously into odontoblast-like cells *in vitro*. The odontoblasts present in the dental pulp may be able to form hard tissue. It is well known that odontoblasts form third dentin after physiological external stimuli to the pulp. If odontoblasts from the dental pulp can be isolated easily and proliferate quickly, the cells will be highly effective for the regeneration of teeth. Undifferentiated mesenchymal stem cells in the dental pulp may also differentiate into odontoblasts. Isolation of undifferentiated mesenchymal stem cells from dental pulp cells would be impossible. In the primary culture, some of these dental pulp-derived cells attached to the culture plate differentiate into odontoblasts or the cells with the ability to form hard tissues. If they proliferate quickly *in vitro*, these pulp derived cells may be used for tooth regeneration. It is desirable for a tooth to be regenerated as the result of differentiation and proliferation of odontogenic cells, such as dental pulp-derived cells. In basic studies for tooth regeneration, it is difficult to obtain and use human dental pulp-derived cells. Thus, rat femoral bone marrow cells (rBMCs) have often been used for the regeneration of hard tissue in teeth [12, 13]. Since the use of bone marrow cells for the regeneration of the teeth was considered possible, femur-derived stem cells have been used in experiments for tooth regeneration. However, it is difficult to obtain bone marrow cells clinically. Therefore, tooth regeneration by dental pulp-derived cells should be emphasized according to clinical circumstances. For this purpose, we investigated the regeneration of teeth using dental pulp-derived cells in order to clarify the culture periods for hard tissue formation.

The necessity of dexamethasone (Dex) in the culture medium was previously reported, and the subculture period for calcified nodule formation by bone marrow-derived cells was found to be 7 to 10 days. Moreover, proliferation and differentiation of cells originating from dental pulp tissue may be slow. Thus, we also evaluated the necessity of Dex in the culture medium during the subculture for hard tissue formation by dental pulp-derived cells.

In this study, we clarified the culture periods required for the proliferation and differentiation of dental pulp-derived cells for teeth regeneration. Then, we compared 20- and 33-day periods regarding the sufficient subculture time for rat dental pulp cells (rDPCs) to form calcified nodules.

2. MATERIALS AND METHODS

2.1. Experimental Animals

In this study, 6-week-old male Fischer 344/N Slc rats (Japan SLC, Inc, Shizuoka, Japan) were used. The Animal Welfare Committee of Osaka Dental University approved the experimental procedures for the use and care of animals. This study was performed at the Laboratory Animal Facilities at the Institute of Dental Research under the Guidelines for Animal Experimentation of Osaka Dental University. Male Fischer 344 rats were kept in standard rat cages with free access to dry pellets and water with unrestricted movement at all times during feeding until experimental use.

2.2. Isolation and Preparation of the Cell Suspension

In this *in vitro* study, to prepare the rDPC suspension, four 6-week-old male Fischer 344/N Slc rats (Japan SLC Inc.) were used. After euthanasia by isoflurane (Forane®; Abbott Japan Co. Ltd., Tokyo, Japan) overdose, the incisors were removed from the transected mandibular bone of the rats. The incisors

were sterilized by soaking in povidone iodine solution (Povidone-Iodine Solution 10%: Meiji Seika Pharma Co., Ltd., Tokyo, Japan) for several seconds and were then washed three times in phosphate-buffered solution without Ca^{2+} and Mg^{2+} (PBS (-); FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Dental pulp tissues from the root canal were pulled out from the pulp canal using a #30 K-type file (Kavo Dental Systems Japan Co., Ltd., Tokyo, Japan) for endodontic root canal treatment. To disperse the rDPCs, the pulp tissue was digested in a trypsin-EDTA solution (0.25 w/v % trypsin and 1 mmol/l EDTA-4Na solution; FUJIFILM Wako Pure Chemical Corp.) in a well of a multi-well culture plate (6-well: BD Biosciences, MA, USA). The plate was placed in an incubator (5% CO_2 and 95% relative humidity at 37˚C) for 30 minutes. The dispersed rDPCs in the trypsin solution containing dental pulp tissue fragments of incisor teeth were isolated by passing through a cell strainer (Pore size: 40 μm, Corning Inc., NY, USA). Trypsin was deactivated by adding culture medium (MEM: Eagle's minimal essential medium: FUJIFILM Wako Pure Chemical Corp.) with 15% fetal bovine serum (FBS; SAFC Biosciences, Inc., KS, USA) and antibiotics (100 U/ml of penicillin, 100 mg/ml of streptomycin and 0.25 mg/ml of amphotericin B; Sigma-Aldrich Co. LLC., MO, USA). MEM was used as the culture medium for rDPCs in this study.

The cells were suspended in 20 ml of MEM in a centrifuge tube (50 ml; Conical centrifuge tube: Corning Inc.) and washed 3 times with MEM by centrifugation at 120 ×g for 10 minutes at 4˚C. After washing, the cells were re-suspended in 18 ml of MEM. The suspension was divided into six 3-ml aliquots, which were poured into separate wells of a cell culture flask (T-25; Corning Inc.) for primary culture in an incubator in 5% CO_2 and 95% relative humidity at 37˚C. The MEM was changed after 24 hours to remove non-adherent cells. The primary culture was performed until the tooth pulp-derived cells became confluent in T-25. The MEM was renewed 3 times per week. After the primary culture period, the rDPCs in the T-25 were washed three times with PBS (-) solution. The cells were released from the bottom of the T-25 culture flasks with trypsin-EDTA solution (0.05 w/v % trypsin and 0.53 mmol/l solution; FUJIFILM Wako Pure Chemical Corp.). The harvested cells were centrifuged at 120 ×g for 5 minutes at 4˚C and re-suspended in the prepared culture medium at 0.5×10^5 cells/ml.

2.3. The Subculture of rDPCs for Calcified Nodule Formation

rDPCs were passaged in multi-well culture plates (6-well) for nodule formation. In each well of the 6-well culture plates, 2 ml of the rDPC suspension at 5×10^4 cells/ml was poured. In two of the plates, the cells were cultured for 20 days. In the other two plates, they were cultured for 33 days. To estimate rDPC differentiation and calcified nodule formation by the cells in each well, 20 μl each of 10 nmol Dex (Sigma-Aldrich Co. LLC.,), 1 mmol β-glycerophosphate (β-GP; EMD Biosciences, Inc., CA, USA) and 82 μg/ml ascorbic acid (Vc; Sigma-Aldrich Co. LLC), respectively, was added to the MEM. As the negative control, rDPCs were cultured in MEM with β-GP but without Dex and Vc. The MEM was renewed three times each week. Calcified nodule formation was confirmed under a phase-contrast inverted microscope (ECLIPSE Ts2: Nikon Corporation, Tokyo, Japan).

2.4. Measurement of the Alkaline Phosphatase Activity Level in rDPC Culture

The buffer solution (TNE: pH 7.4), which consisted of 1 mmol of 2-Amino-2-(hydroxymethyl)-1, 3-propanediol hydrochloride (FUJIFILM Wako Pure Chemical Corp.), 0.1 mmol of ethylene-di-amine tetra-acetic acid tetra-sodium tetra-hydrate salt (FUJIFILM Wako Pure Chemical Corp.) and 10 mmol of sodium chloride (FUJIFILM Wako Pure Chemical Corp.), was prepared for the biochemical analysis of alkaline phosphatase (ALP) activity and quantitative analysis of Ca^{2+}. All the procedures for analysis of ALP activity were performed at 3˚C to 6˚C.

After passaging, each cell layer in the wells containing deposited calcified nodules was washed three times with PBS(-). Then, the layer was scraped off with the calcified nodule and collected in 500 μl of TNE buffer solution in a conical micro centrifuge tube. The cells in 500 μl of TNE buffer solution were sonicated (BIORUPTOR UCW-201; Tosho Denki Co., Ltd., Yokohama, Japan) for 30 seconds at 3˚C. For DNA measurement, 100 μl of the supernatants was mixed with 200 μl of Hoechst 33258 (FUJIFILM Wako

Pure Chemical Corp.) at 2.5 μg/ml. The amount of DNA was measured using a fluorescence-spectrum photometer (Spectra-Max M5; Molecular Devices, Inc., CA, USA) at an excitation wavelength of 355 nm and fluorescence emission at 460 nm. After the quantitative analysis of DNA, the sonicated cell suspension was centrifuged at 18,000 ×g for 1 minute. To measure ALP, 100 μl of *p*-nitrophenyl phosphate (PNP: Thermo Fisher Scientific Inc., MA, USA) was added as a substrate to 20 μl of the supernatant and incubated at 37˚C for 30 minutes. The reaction was stopped by adding 100 μl of sodium hydroxideat a concentration of 0.2 mol. The amount of *p*-nitrophenol produced by the reaction of the supernatant with PNP was measured using the absorbance at a wavelength of 405 nm with a fluorescence-spectrum photometer (Spectra-Max M5). Salmon sperm deoxyribonucleic acid (DNA; Thermo Fisher Scientific Inc., MA, USA) was used as the standard. It was diluted to 5, 10, 25, 50 and 100 μg/ml in the TNE solution to obtain a calibration curve.

ALP activity, as the ALP/DNA ratio, is presented as μmol of *p*-nitrophenol released after 30 minutes of incubation at 37˚C. The results are expressed as the mean ± standard error. Statistical comparisons between the mean values were performed using two-way unrepeated ANOVA followed by post hoc analysis using the Tukey-Kramer's test. Differences of $p < 0.01$ were considered significant.

2.5. Measurement of Osteocalcin in rDPC Culture Supplemented with and without Dex

Enzyme-immunoassay for the quantitative determination of osteocalcin (OC) in the supernatant of rDPC culture medium collected at 20 days or 33 days from the wells of all culture plates was performed. The quantity of OC in the supernatant was measured immunochemically using a commercially available rat OC ELISA kit (Rat-MID™ Osteocalcin ELISA, Medical & Biological Laboratories Co., Ltd., Aichi, Japan). All procedures of the assay were performed at room temperature (18˚C - 22˚C) according to the manufacturer's instructions. In the 96-well micro plate, 20 μl of unknown samples was pipetted into the wells followed by 150 μl of the mixture of the antibody in incubation buffer, and incubated for 60 minutes at room temperature. Then, 100 μl of the substrate solution was added into each well and incubated for 15 minutes at room temperature in the dark. The reaction in each well was stopped by adding 100 μl of 0.18 mol sulfuric acid. Absorbance at 450 nm was measured using a spectrophotometer (Spectra-Max M5).

These results are shown as the mean and standard error. Statistical comparisons between the mean values were performed using two-way unrepeated ANOVA followed by post hoc analysis using the Tukey-Kramer's test. Differences of $p < 0.01$ were considered significant.

2.6. Quantitative Analysis of Ca^{2+} from Calcified Nodules in rDPC Culture

Calcified nodules were produced in the culture medium by rDPCs with the addition of Dex. The quantity was expressed as a quantity of Ca^{2+}. The sonicated and centrifuged precipitate of the scraped cell layer with calcified nodules from each well of the cell culture plate was poured into a 1.5-ml centrifugation tube and centrifuged at 16,000 ×g for 10 minutes. Decalcification of the precipitate was performed in the tube containing 500 μl of a 20% formic acid solution for 5 days using a laboratory shaker at 4˚C. After decalcification, the tubes were centrifuged at 1600 ×g for 10 minutes.

To calculate the relative quantity of developed nodules in the culture of rat incisor pulp cells, the amount of Ca^{2+} in the precipitate was measured. For the quantitative analysis of Ca^{2+}, a commercially available kit (Calcium E-test Wako®: FUJIFILM Wako Pure Chemical Corp.) was used. The outline of the assay is as follows: A mono-ethanolamine buffer (pH 12) and methyl xylenol blue as the coloring reagent were included in the kit. After centrifugation, 50 μl of the supernatant was added to 2 ml of the buffer solution. Methyl xylenol blue binds to Ca^{2+} under alkaline conditions, and the reactant turns blue. Then, the Ca^{2+} produced in the cell culture was measured by the absorbance at 610 nm using a fluorescence-spectrum photometer.

The results are expressed as the mean ± standard error. Statistical comparisons between the quantities of Ca^{2+} were performed using two-way unrepeated ANOVA followed by post hoc analysis with Tukey-Kramer's test. Differences of $p < 0.01$ were considered significant.

3. RESULTS

3.1. Phase-Contrast Microscopic Examination of Calcified Nodule Formation in the Cell Culture

The cultured rDPCs were observed under a phase-contrast microscope. At the 20-day observation period of the subculture, little calcified nodule formation was noted with or without Dex. In the cell culture with Dex, calcified nodules were observed at 33 days of subculture (**Figure 1(a)**). The calcified nodules formed in culture plates exhibited a low degree of calcification. Without Dex in the culture medium, no calcified nodules were present (**Figure 1(b)**).

3.2. ALP Level in rDPC Culture

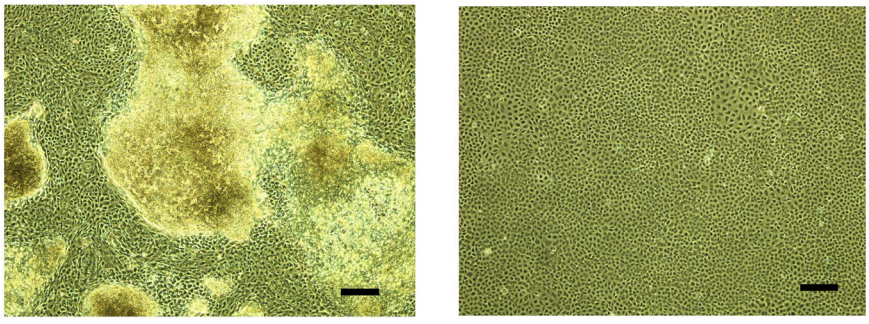
The ALP level in rDPC culture for 20 days and 33 days is shown in **Figure 2(a)**. The ALP level in the 20-day rDPC culture with Dex was 2.46 ± 0.25 μmol/ml and it was 0.98 ± 0.02 μmol/ml without Dex. There was a significant difference ($p < 0.01$). The ALP level in the 33-day culture without Dex, at 1.08 ± 0.05 μmol/ml, was also different from the level in the 20-day culture with Dex. The level of ALP in the 33-day culture without Dex was not significantly different from the 20-day culture without Dex ($p < 0.01$). The rDPCs cultured for 33 days in the medium containing Dex exhibited a significantly high ALP level of 22.30 ± 0.76 μmol/ml.

3.3. ALP Activity of rDPCs Culture

ALP activity of rDPCs in 6-well culture plates for 20 days and 33 days is shown in **Figure 2(b)**. The culture of rDPCs for 20 days with and without Dex demonstrated low activity. There was no significant difference in ALP activity in the 20-day culture between with and without Dex ($p < 0.01$). With Dex, significantly high ALP activity was observed in the 33-day culture of rDPCs, at 0.56 ± 0.02 μmol/μg of DNA. However, the 33-day culture of rDPCs without Dex in the medium had low ALP activity ($p < 0.01$).

3.4. Quantity of OC in rDPC Culture

The quantity of OC in rDPC culture for 20 or 33 days is shown in **Figure 3**. There was no significant difference in the quantity of OC among the culture of rDPCs for 20 days with and without Dex, and for 33 days without Dex. The quantity was approximately 0.33 - 0.84 ng. The measured quantity in the 33-day culture with Dex was 18.77 ± 1.23 ng. This was significantly higher than that in the culture for 20 or 33 days without Dex.



(a) (b)

Figure 1. (a) Phase-contrast microscopic examination of calcified nodule formation in the culture medium with Dex. The 33-day subculture period. In the dental pulp cell culture with Dex, calcified nodules were observed (Bar: 200 μm); (b) Phase-contrast microscopic examination of calcified nodule formation in the culture medium without Dex. The 33-day subculture period. The proliferating dental pulp cells were confluent. No calcified nodules were present (Bar: 200 μm).

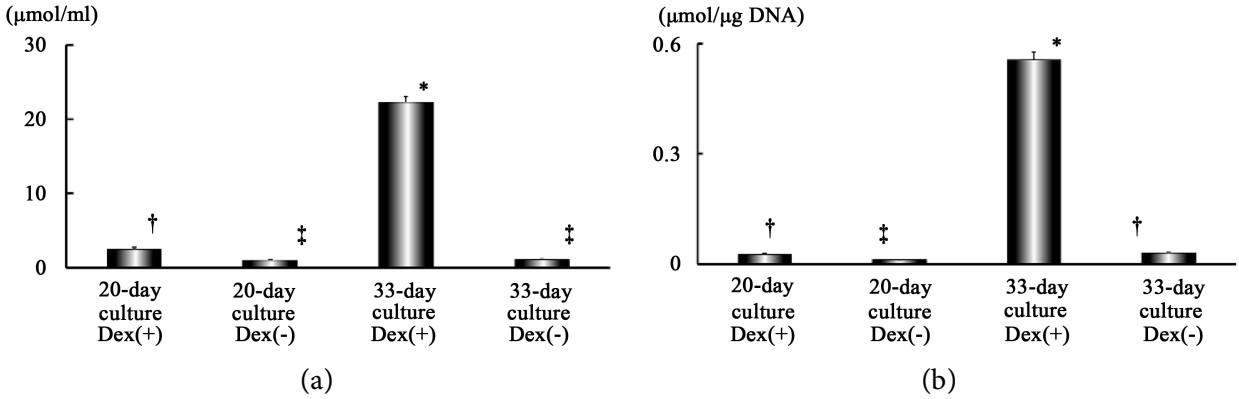


Figure 2. (a) ALP level measured in rDPC culture for 20 or 33 days with and without Dex. The ALP level measured in the 20-day rDPC culture with Dex (†) was significantly different from that in the culture without Dex (‡). Without Dex in the subculture, there was no difference in the level of ALP in the 33-day culture and 20-day culture. The rDPCs cultured for 33 days in the medium with Dex (*) had a significantly high ALP level ($p < 0.01$); (b) ALP activity of rDPCs cultured for 20 or 33 days with and without Dex. The culture of rDPCs for 20 days with and without Dexexhibited low ALP activity. However, there was significant difference between the 20-day culture with (†) and without (‡) Dex. With Dex, ALP activity in the 33-day culture of rDPCs was significantly high (*). Without Dex in the medium, the 33-day culture of rDPCshad low ALP activity (†) ($p < 0.01$).

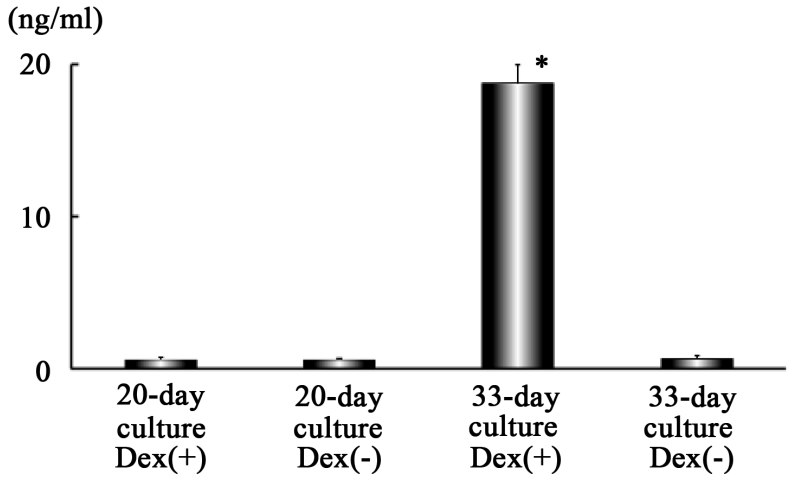


Figure 3. Quantity of OC in rDPC culture for 20 or 33 days with and without Dex. The quantity of OC in rDPC culture for 33 days without Dex was not significantly different from that in rDPC culture for 20 days with and without Dex. The quantity of OC in the 33-day rDPC culture with Dex (*) was significantly higher than that in the culture for 20 days or 33 days without Dex ($p < 0.01$).

3.5. Quantity of Ca^{2+} from Calcified Nodules

We next measured the quantity of Ca^{2+} in the 20- or 33-day culture of rDPCs in the medium with and without Dex. The level after decalcification of the nodules was significantly high in the 33-day culture with Dex, being 45.01 ± 1.13 mg/dl (Figure 4). The other cultures of rDPCs for 20 days with or without Dex and for 33 days without Dex had levels of Ca^{2+} of 13.4 - 13.9 mg/dl. There was no significant difference ($p < 0.01$).

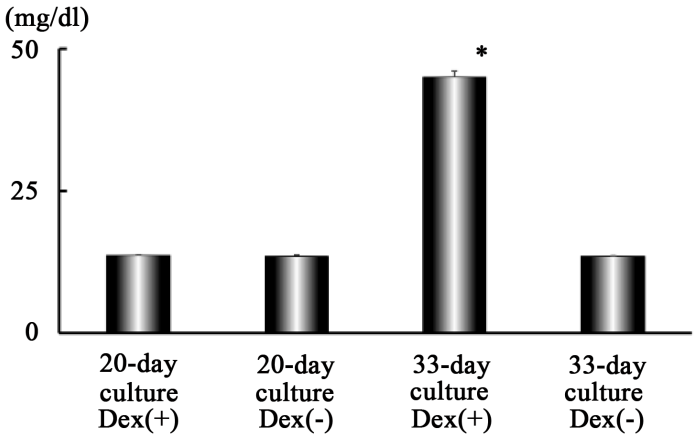


Figure 4. Amount of Ca^{2+} from calcified nodules. The amount of Ca^{2+} after decalcification of nodules in the culture of rDPCs for 33 days with Dex(*) was significantly high. The cultures of rDPCs for 20 days with or without Dex and for 33 days without Dex were not significantly different ($p < 0.01$).

4. DISCUSSION

In contrast to the healing of skin and bones, autonomous repair of damaged dentine or dental pulp is difficult. In hard tissues of teeth, defects cannot be reconstructed by newly formed hard tissue. The dentine that is decalcified by caries must be removed by dental treatment. As a result, defects occur in hard tissues of teeth. Inflamed or gangrenous dental pulp should also be removed. After extirpation of damaged dental pulp, a cavity in the tooth is made through root canal treatment. The cavity must be filled with an artificial material to prevent re-infection. Although the lesions in the pulp or periapical region were treated, the possibility of dental caries or periapical periodontal diseases remains. This is due to the presence of a micro-flowing passage between the marginal edge of the material and the margins of the cavity.

A bridge or denture is applied for lost teeth. Treatments using artificial products, such as dentures, crowns, bridges or oral implants, can recover tooth function, but there are no biological treatments for teeth. Physiological reconstruction is desirable for a tooth with a partial defect [14]. It may be difficult *in vitro* to regenerate the whole tooth consisting of dentine, including dental pulp, cementum and enamel. The use of a tooth germ has been considered suitable for regeneration of a complete tooth [15], and the tooth germ, which ectopically autografted in a mouse, matured to a complete tooth [16]. However, this method places a heavy surgical burden on the patient to obtain a tooth germ from alveolar bone. Thus, tooth regeneration using pluripotent stem cells originating from any tissue in the oral cavity is desirable [17]. The isolation of the stem cells from dental pulp was first reported by Gronthos *et al.* [18], and stem cells originating from human deciduous teeth were also previously reported [19, 20].

Dentine, such as reparative dentine, will be formed by odontoblasts in the dental pulp of a viable tooth. Therefore, hard tissue may be induced autonomously by the odontoblasts contained in the dental pulp-derived cells. However, calcified nodules by the dental pulp-derived cells were not formed autonomously in this study. One possibility is the dedifferentiation of the dental pulp cells into stem cells *in vitro*. It is considered that there were few odontoblasts among the cells in the dental pulp extracted from the mandibular incisor teeth of rats. There may be some mechanism in the pulp for dedifferentiating the dental pulp cells into blast cells. Such mechanisms were not reproduced in this *in vitro*. Based on our *in vitro* preliminary experiments, the period for dental pulp cell culture until calcified nodule formation from the primary culture may be long. In the previous *in vivo* and *in vitro* studies by Yoshikawa *et al.* [5] [21-23], bone marrow cells from the femur of rats were used to examination hard tissue formation as a substitute for cells obtained from dental pulp. The proliferation of stem cells in dental pulp cells was not evaluated and osteogenic potential of bone marrow cells was not compared with hard tissue forming ability by dental pulp-derived cells in this study. It was judged that several stem cells should have grown together in it when

the confluency of dental pulp cells was confirmed in the primary culture.

Osteoprogenitor cells were reported to account for approximately 70% of mesenchymal stem cells originating from human bone marrow [24]. Park *et al*. compared the proliferation and multilineage differentiation of the cells from the periosteum and bone marrow of mandibular bone and the tibia using rabbit models. They found that the cells from the periosteum of the jaw bone had a higher ability than bone marrow-derived cells [25]. On the other hand, it was also reported that the number of proliferating cells as well as the frequency of colony-forming cells were significantly higher among dental pulp stem cells when compared with bone marrow stromal stem cells [26]. The dental pulp cells induced from human deciduous tooth demonstrated high proliferation capability [27]. However, one problem with the use of DPCs is the small number of cells available because of the small size of the pulp cavity. Smith *et al*. reported that the number of stem cells contained in the dental pulp is less than 1% of the total cell number [28]. The report by Smith *et al*. supports our results. When rat bone marrow cells were used, the calcified nodules formed within a shorter period than when using dental pulp cells. In this study, a longer period was needed for calcified nodule formation by dental pulp-derived cells. This study suggested that the population of stem cells in dental pulp is very small. Indeed, the number of bone marrow cells collected from the rat femur was larger than that from rat mandibular incisors. This was confirmed by comparing the capacity between the rat femur and the pulp cavity of the mandibular incisor. There are many types of stem cells in high quantities in the bone marrow.

In the report by Matsui *et al*. [29] on the mineralization potential after differentiation of human dental pulp stem cells from the third molar of a young patient, the experimental period after adding Dex to the culture medium was 14 - 21 days. The proliferative period in the primary culture of stem cells obtained from dental pulp was not described in most reports. In the report by Kumabe *et al*. [30], the period required for the culture of human pulp cells was 2 months comprising 13 passages. In comparison with the bone marrow cells from rat femurs, a longer culture period for rat dental pulp cells may be necessary to form the calcified nodules *in vitro*. At least a 3-week period of continuous treatment of a confluent monolayer of cells with Dex, in combination with β-GP and Vc, was reported to be required for osteogenic differentiation [31]. In general, 10 days of primary culture followed by 10 - 14 days of subculture is considered to be required to obtain calcified nodules *in vitro* using rBMCs from the femur. In our previous *in vitro* studies using rat bone marrow cells, the period required to form calcified nodules was 1 week for primary culture and 2 weeks of subculture [22]. In this *in vitro* study, calcified nodule formation by rDPCs was examined at 33 days. Although calcified nodules were slightly observed on the culture plate several days before day 33, these results suggested that there were few stem cells in the dental pulp.

Usually, osteogenic examination *in vivo* and *in vitro* using rat bone marrow cells, the cells are cultured to be confluent in primary culture. And, the rBMCs are cultured in the medium containing Dex. In the subculture, some of the stem cells or progenitor cells in the rBMCs differentiate to odontoblasts by the effects of Dex and form bone-like hard tissue. Similarly, hard tissues had been formed by dental pulp cells with addition of Dex in MEM in this study. However, the differentiation situation of stem cells in the dental pulp would not be cleared. As same as in bone, osteocalcin is detected as a specific index in dentine. Dental pulp cells were prepared as same as bone marrow cells in our study. Hard tissue formation will be induced by the stem cells in the dental pulp. The cells might be differentiated into osteoblasts by Dex in the subculture.

In this study, calcified nodules were formed by cells from dental pulp. The appearance of the nodules was different from those formed by rBMCs in our previous reports [21-23]. Microscopically, the calcified nodules formed by rDPCs had lower levels of Ca^{2+} deposition than the nodules by rBMCs. It is well known that calcified deposits are formed *in vitro* by cells from bone marrow. Cells originating from the bone marrow ectopically form cartilage *in vivo*, and because the level of calcification is lower than that in bone, the product should be regarded as cartilage. The calcified nodules generated by cells differentiated from stem cells derived from pulp may also have produced lower levels of Ca than nodules by bone marrow-derived stem cells. This study suggested that the type of hard tissue-forming cells used for tooth regeneration affects the hardness of hard tissue.

5. CONCLUSIONS

The purpose of this study was to clarify the culture periods required for the proliferation and differentiation of dental pulp-derived cells for tooth regeneration. Based on this study, the following conclusions were obtained.

A long primary culture period for the dental pulp cells is required for proliferation of a sufficient number of cells to form calcified nodules in the secondary culture. Based on this, the following conclusions were obtained.

Dexamethasone was found to be essential for the formation of mineralized aggregates by dental pulp-derived cells.

Formation of calcified aggregates by dental pulp-derived cells *in vitro* required more than one month. The duration of the secondary culture for the dental pulp-derived cells is much longer than that of bone marrow-derived cells.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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