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(RESEARCH ARTICLE)

Transferrin promotes calcified nodule formation in rat bone marrow cell culture with dexamethasone

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Abstract

Some chemicals can promote the differentiation of stem cells and the formation of hard tissues. In this study, the effects of transferrin on calcified nodule formation were evaluated using bone marrow cells obtained from the femora of Fischer 344 rats. Transferrin was added to the culture medium to promote the proliferation and differentiation of stem cells among the bone marrow cells into osteoblasts or chondroblasts. Calcified nodule formation was confirmed macroscopically in the culture medium and evaluated quantitatively by measuring Ca^{2+} in the solution after demineralization using formic acid. The cells were cultured in 2 ml of minimum essential medium with 20 µl of solution containing 100, 200 or 400 ng of transferrin. Dexamethasone was also added to the medium at 10 nmol. Subculturing was performed for 2 weeks. The concentration of Ca^{2+} after decalcification of the calcified nodule formed in the bone marrow cell culture with dexamethasone was approximately 10 µg/ml. The addition of 100~200 ng of transferrin to the bone marrow cell culture resulted in the maximum concentration of Ca^{2+} and size of the formed calcified nodule in the medium. For calcified nodule formation by bone marrow cells, the optimal concentration of transferrin in the culture medium was suggested to be 200 ng in 2 ml. This study suggested that transferrin is beneficial together with dexamethasone. It may play an important role in bone formation *in vivo*.

Keywords: Transferrin; Dexamethasone; Calcified nodule; Bone marrow cells; *In vitro*

1. Introduction

Chemicals, such as dexamethasone (Dex), β-glycerophosphate (β-GP) and bone morphological proteins, have been used as factors to promote the differentiation of stem cells and the formation of hard tissues *in vitro*. Such chemicals should have no antigenicity, and must not be harmful to the tissues and organs. Currently, addition of Dex, β-GP and ascorbic acid (Vc) to the medium as inducers is essential for *in vitro* bone regeneration using stem cells [1]. Many cells originating from dental pulp or bone marrow are needed for bone formation. Regeneration of bone or dentin by these differentiated cells requires a considerably long period of time. To obtain many osteogenic cells from the stem cells through the differentiation processes, some factors should be important for differentiation of the cells or osteogenesis.

The iron content may be closely related to bone formation [2]. Transferrin (Tf) transports iron through the body via blood because all cells require iron to take oxygen from their surroundings. Tf is one of the chondrogenic stem cell differentiation inducers that functions with ascorbic acid, insulin, selenite and transforming growth factor-β1 [3]. It is commercially available as a cell growth factor. The Tf receptor is a homodimeric transmembrane glycoprotein that binds to iron-bound Tf [4] and regulates extracellular iron to be delivered to cells via clathrin-dependent endocytosis [5]. Thus, iron is an essential factor for cell growth and activity maintenance.

It was previously reported that Tf-receptor-positive cells differentiated into osteoblasts by tissue engineering for bone regeneration in the presence of inducers in the medium [6]. It is well known that there are many stem cells that

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differentiate into osteoblasts in the bone marrow. Tf may affect bone marrow cells *in vitro* as one of the factors for stem cell differentiation into osteoblasts. The involvement of Tf in hard tissue formation may be due to the presence and the function of the Tf receptor on the cell because it is a glycoprotein that binds to iron-bound Tf and regulates the extracellular iron to be delivered to cells [5]. Although Tf is widely believed to be important for iron acquisition by all mammalian cells, its physiological function for mammalian development is poorly understood. Moreover, a negative effect of bone restriction on the function of osteoblasts and bone formation in rats was previously reported [7]. Other studies found that differentiation into osteogenic blast cells and osteoblast activation were promoted by iron chelation [8, 9]. A recent study revealed that low iron levels increase osteoblast activity, whereas low levels of iron inhibit osteoblast activity [10]. As described above, there are many considerations on the effects of iron on the process of differentiation of stem cells into osteoblasts and subsequent bone formation.

In dentistry, less invasive cell sources from which stem cells can be obtained are limited. They consist of pulp or oral mucosa, or the tooth root membrane of extracted teeth. As there are few cells in the pulp, few stem cells will be obtained. It will also take a long time before they proliferate and differentiate into osteoblasts, and until bone formation. Differentiation into osteoblasts is difficult for stem cells obtained from mucosal cells, which are ectodermal. In order to differentiate these stem cells into osteoblasts and for odontoblasts to form bone or dentin, it is important to clarify the factors that induce the rapid proliferation and differentiation of stem cells. Therefore, we added Dex and Tf to the culture solution for stem cell bone formation *in vitro* to evaluate novel effects during the process of differentiation into blast cells, leading to hard tissue formation.

Bone formation by cells originating from bone marrow may be promoted when Tf is added as a co-factor in medium supplemented with essential osteogenic inducers. We hypothesized that calcified nodule formation will be promoted by the stem cells originating from bone marrow by the addition of Tf, Dex, β-GP and Vc to the subculture of rat bone marrow cells (rBMCs). Therefore, Tf was examined as a co-factor of Dex in this study for its effects on differentiation and induction of bone formation. Calcified nodule formation by rBMCs from femora by the addition of Tf to the culture medium with Dex was evaluated *in vitro* by measuring the amount of Ca^{2+}.

2. Material and methods

2.1. Animals

This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University. Regarding the use and care of the animals, the Animal Welfare Committee of Osaka Dental University approved the experimental procedures. Male Fischer 344 rats (Clea Japan, Inc., Tokyo, Japan) were used in this study. The rats were kept in standard rat cages with free access to dry pellets and water and unrestricted movement at all times during feeding.

2.2. Rat bone marrow cell preparation

From the shafts of the femora of 6-week-old male Fischer 344 rats, rat bone marrow cells (rBMCs) were extracted after euthanasia by excessive inhalation of isoflurane (Forane®; Abbott Japan Co. Ltd., Tokyo, Japan). Both ends of the femur were cut off at the epiphysis, and bone marrow was flushed out using 10 ml of culture medium expelled from a syringe with a 21-gauge needle. Primary culture of the cells was performed for 1 week in a cell culture flask (T-75; BD Biosciences, MA, USA) containing culture medium (MEM: Eagle's minimum essential medium containing L-glutamine and phenol red: FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with 15% fetal bovine serum (FBS; Biocera Inc., MO, USA) and antibiotics (100 unit/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B; Sigma-Aldrich Co., MO, USA). The medium was changed 2 times.

After primary culture, rBMCs in the T-75 culture flask were washed three times with phosphate-buffered solution without Ca^{2+} and Mg^{2+} (PBS (-); FUJIFILM Wako Pure Chemical Corp.). To prepare the rBMC suspension, the cells were isolated from the bottom of the T-75 flask with 2 ml of trypsin-EDTA solution (0.05 w/v% Trypsin-0.53 mmol/l of EDTA-4Na solution with phenol red: FUJIFILM Wako Pure Chemical Corp.) and incubated for 1 minute at 37˚C. The cells attached to the bottom of the T-75 flask were dispersed in MEM by shaking the flask. To stop the effects of trypsin, 8 ml of MEM containing FBS was poured into the flask. The cells were transferred to a centrifuge tube and washed three times by centrifugation at 120 × g using 20 ml of PBS (-). Then, to prepare the rBMC suspension, the cells were re-suspended in MEM at 0.5×10^5 cells/ml for examination.

2.3. Culture of rBMCs in the medium with Tf solution

Tf (FUJIFILM Wako Pure Chemical Corp.) solution was prepared at 5, 10 or 20 μg/ml in MEM without FBS or antibiotics.

rBMCs were seeded in 2 ml of MEM at 1 × 10^4 cells/ml in each well of 6-multi-well culture plates (BD Biosciences). In each well of the plates, 20 µl each of 10 nmol of dexamethasone (Dex; Sigma-Aldrich Co.), 1 mmol of β-glycerophosphate (β-GP; EMD Biosciences, Inc., CA, USA) and 82 µg/ml of ascorbic acid (Vc; Sigma-Aldrich Co.) was added. Each time, 20 µl of 5, 10 or 20 µg/ml Tf solution was added to assess its effects on calcified nodule formation in the wells. As a control, Dex, β-GP and Vc without Tf solution were added to the MEM for rBMC culture. The addition of Dex with β-GP and Vc is indispensable for hard tissue induction. Therefore, in this study, β-GP and Vc were always added with Dex. rBMCs were also cultured in MEM with 20 µl of β-GP and Vc as a negative control. The subculture for calcified nodule formation was performed for 11 days. The MEM was changed every 2 days. For each cell culture condition, 6 wells were used.

2.4. Measurement of ALP activity and quantitative analysis of Ca2+

The supernatant in each well of all culture plates was collected after subculture for 11 days for the biochemical analysis of alkaline phosphatase (ALP) activity and quantitative analysis of Ca^{2+}. Each cell layer in the wells containing deposited calcified nodules was washed three times with PBS(-).

For quantitative analysis of ALP, 1 ml of TNE buffer solution (pH 7.4) was poured into each well, and the subcultured cell layer on the plate was scraped off and transferred into a 1.5-ml micro tube. The TNE buffer solution 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.).

The cells in the buffer solution were sonicated (BIORUPTOR UCW-201; Tosho Denki Co., Ltd., Yokohama, Japan) for 30 seconds at 3˚C. Aliquots of 0.1 ml of the sonicated cell suspensions were used for quantitative analysis of DNA. Salmon sperm DNA (Life Technologies Inc., CA, USA) was used as the standard. DNA was measured by fluorescence emission in the presence of 2.5 µg/ml of Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan). 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. The remaining sonicated cell suspension in the micro tube was used to measure ALP level. The suspension was centrifuged at 16,000 × g for 1 minute. To measure the ALP level, 0.1 ml of *p*-nitrophenol phosphate (PNP: Zymed Lab.) was added as the substrate to 20 µl of the supernatant and incubated at 37˚C for 30 minutes. The reaction was stopped by adding 100 µl of NaOH at a concentration of 0.2 mol. The amount of *p*-nitrophenol produced by reaction of the supernatant with PNP was measured by the absorbance at a wavelength of 405 nm with a fluorescence-spectrum photometer (Spectra-Max M5). The ALP level in the cultured bone marrow stem cells with and without Tf solution in the culture medium was represented as µmol of *p*-nitrophenol released after 30 minutes of incubation at 37˚C. The ALP/DNA ratio was calculated as the ALP activity. The amount of calcium in each well was measured using the commercially available Calcium-E test WAKO® (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). The amount of calcified nodules produced in the culture medium by rDPCs was expressed as a quantity of Ca^{2+}. Each precipitated calcified nodule in the micro tube was decalcified by adding 1 ml of 20% formic acid for 72 hours using a laboratory shaker at 4℃. After decalcification, the tubes were centrifuged at 1600 ×g for 10 minutes.

Data are presented as the mean ± standard deviation. Statistical comparisons between the mean values of ALP activity and amount of Ca^{2+} in each well were performed using two-way unrepeated ANOVA followed by post hoc analysis using the Tukey-Kramer test. Differences of $p < 0.01$ were considered significant.

3. Results

3.1. Measurement of ALP activity

The amount of ALP from cultured rBMCs in the well after the 11-day subculture was 0.232 ± 0.011 mmol/l after adding Dex to the medium. There was no significant difference in the amount in rBMCs cultured with Dex among the varying concentrations of Tf. In cells without Dex, the level was approximately 0.121 mmol/l, being significantly different.

The DNA concentration in the rBMC culture without Dex was 10.224 ± 0.656 µg/ml. The amount of DNA in the other cells was 9.952 µg/ml to 10.202 µg/ml on average. There was no significant difference between cells with and without Dex or Tf in the medium.

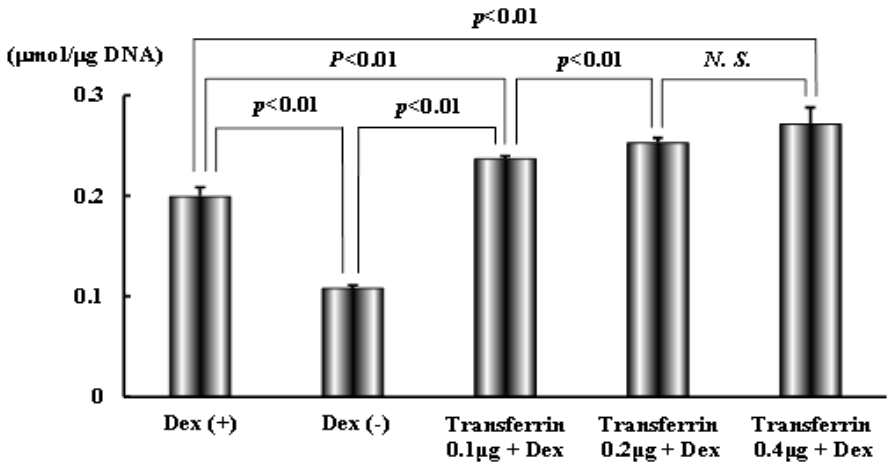

Figure 1 ALP activity in rBMCs cultured in the medium

The addition of Dex to the medium induced higher ALP activity in rBMCs as a positive control than that in the negative control. The addition of transferrin to the medium affected the activity of rBMCs.

Based on the quantitative analysis of ALP and DNA, the ALP activity of rBMCs cultured in the medium under each condition was calculated. A significant difference was noted between the cells cultured with and without Dex. For rBMCs cultured in medium supplemented with Dex, the concentration of DNA in cells with 0.2 μg of Tf was 25.814 ± 0.777 μmol/μl. This was significantly different from the 24.590 ± 0.344 μmol/μl of DNA in the cells cultured with 0.1 μg of Tf and 23.554 ± 0.779 μmol/μl of DNA in the cells cultured without Tf. The ALP activity is shown in Figure 1. This suggested that Tf with Dex increased the activity of cultured rBMCs.

3.2. Quantitative analysis of Ca^{2+} from calcified nodules in rBMC culture

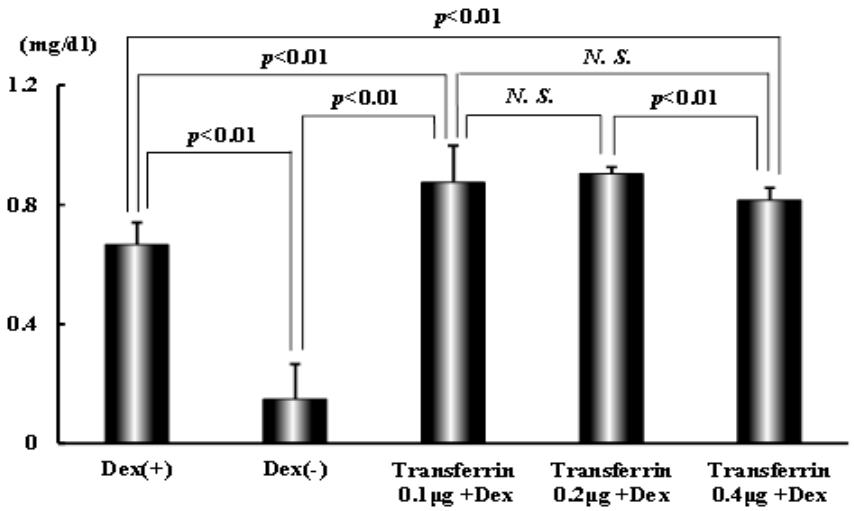

Figure 2 Quantitative analysis of Ca^{2+} from calcified nodules in rBMC culture

rBMCs cultured with Dex as a positive control had a significantly greater amount of Ca^{2+} than the negative control. The amount of Ca^{2+} produced by rBMCs cultured with transferrin and Dex was significantly higher than that by the positive control. In cells cultured with Dex and transferrin, the amount of Ca^{2+} produced after the addition of transferrin at 0.4 μg was significantly different from that after the addition of 0.2 μg of transferrin. (p<0.01)

The amount of Ca^{2+} in the 11-day culture of rBMCs was measured using the calcified nodules in the well after decalcification. rBMCs cultured with Dex as a positive control induced 0.667 ± 0.073 mg/dl of Ca^{2+}. This was significantly different from the 0.904 ± 0.022 mg/dl in the cell culture with Dex and 0.2 μg of Tf. In the medium with Dex and 0.1 μg of Tf, rBMCs induced a significantly high amount of Ca^{2+} of 0.874 ± 0.123 mg/dl (Figure 2). There was no difference between the amount of Ca^{2+} in the medium with Dex and 0.1 μg of Tf and that in the medium with 0.2 μg of Tf. The amount of Ca^{2+} produced from rBMCs cultured with Dex and 0.1 μg of Tf was not significantly different from the 0.815 ± 0.040 mg/dl produced from those cultured with Dex and 0.4 μg of Tf. The amount of Ca^{2+} produced from rBMCs cultured with Dex and 0.2 μg of Tf was significantly different from that from those cultured with Dex and 0.4 μg of Tf. (p<0.01).

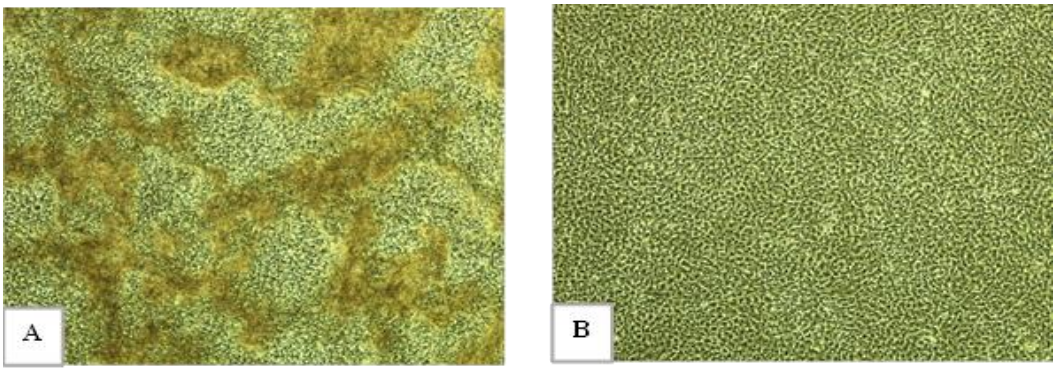

Figure 3 Representative findings after 11-day culture of rBMCs

A. Cultured with Dex and tryptophan in the medium. Among the proliferated rBMCs in the medium, aggregates recognized as calcified nodules in varying shapes were frequently observed. **B.** Cultured without Dex and transferrin. Proliferated rBMCs were observed in the culture plate. No calcified nodules were found.

As shown in Figure 3-A, calcified nodules were observed in the culture of rBMCs with Dex and Tf in the medium. rBMCs cultured for 11 days without Dex as a negative control exhibited no calcified nodule formation (Figure 3-B).

4. Discussion

In the studies of experimental bone formation, Dex, β-GP and Vc have been used as factors for the differentiation of stem cells into osteoblasts [11, 12]. rBMCs from rat femora have been frequently used for the experimental regeneration of hard tissues such as bone [12]. Calcified nodules are formed by osteoblasts in the culture medium in the presence of Dex, β-GP and Vc [13]. In culture medium containing these factors, the stem cells originating from the bone marrow may differentiate into osteogenetic progenitor cells, which should then differentiate into osteoblasts or chondroblasts. Bone or cartilage may be formed by these cells in the medium. For these processes, a long time is needed. Bone-forming cells were reported to require a period of 3-4 weeks or longer to form calcified nodules in the culture medium [14]. Thus, it will take a long time to regenerate hard tissues such as bone and teeth. Methods to reduce this period and to promote the formation of a large amount of hard tissue are required. Therefore, in addition to the use of factors, such as Dex, methods or factors that can shorten the period leading to stem cell proliferation, differentiation and hard tissue formation by differentiated blast cells are desired.

Lactoferrin was reported to activate osteoblasts and induce bone formation [15]. Based on these previous reports, lactoferrin may be appropriate as a co-factor with Dex. In this study, however, Tf was selected as an osteogenic inducer and its effects were investigated using cells originating from bone marrow. Thus, it may be necessary to consider the differences between Tf and lactoferrin in bone formation. The conformation of lactoferrin is similar to that of Tf, which is a protein that transports iron in plasma. However, the affinity of lactoferrin for iron ions is 100-times higher than that for the protein [9]. In other words, lactoferrin captures iron and removes it from the surrounding environment. Similar to Tf, lactoferrin has a functional group that binds strongly to iron, but its main function may be not the supply of iron to cells [16]. Thus, lactoferrin may play no role in iron ion transport because the function of the peptide part is strong. It was previously reported that lactoferrin promotes the proliferation and differentiation of osteoblasts [17], whereas the suppression of bone resorption by osteoclasts promoted osteogenesis [18]. Bone regeneration may be promoted by the administration of bovine lactoferrin [19]. Moreover, local application of lactoferrin promoted bone regeneration in rats [20]. The characteristic of osteogenesis by lactoferrin is that both

the proliferation and differentiation of osteoblasts, and inhibition of bone resorption by osteoclasts progress simultaneously.

The purpose of this study was to investigate whether Tf increases the activity of Dex, which induces the differentiation of and hard tissue formation by stem cells. Tf is a group of glycosylated, iron binding, 80-kD proteins [21]. As iron is an essential trace metal for all cells [22], Tf should be an important factor for the differentiation and proliferation of stem cells in rBMC culture, and for the formation of calcified nodules. The addition of Tf to the culture medium of rBMCs as a co-factor with Dex to promote calcified nodule formation was investigated in this *in vitro* study. The presence of Dex in the medium induces differentiation of stem cells among rBMCs into hard tissue-forming blast cells such as osteoblasts. Tf as the co-factor was hypothesized to increase the effects of Dex. Tf is the main component of serum iron, which is non-heme iron involved in iron transport. Tf-bound iron is transported to the bone marrow via blood [23]. On the other hand, cells isolate and store the necessary amount of iron ions inside and supply them when required. It is known that ferritin and Tf function in this process, and the extra iron ions in the cells that are confined securely in the shell made of ferritin are delivered by Tf to the stem cells, enabling them to differentiate into osteoblasts. It was previously reported that the Tf receptor (p90, CD71) encoded by the TFRC gene was required for iron delivery from Tf to cells [24]. Tf in serum transports iron ions to target cells via specific receptors [5]. Stem cells with the Tf receptor may differentiate into osteoblasts, leading to bone formation [25]. Most of the stimulatory activity of Tf is suspected to be related to its iron-binding properties, and it is well known that Tf-bound iron is transported into cells [6].

Tf, insulin and selenium were added to the medium in many previous *in vitro* studies on bone or hard tissue formation [3, 25]. The mixture of insulin, Tf and selenium has been considered as a general cell supplement designed for use in conventional medium, and it increases cell proliferation and decreases the serum requirement of many cell types. Many reports suggesting the necessity of adding insulin-like growth factor into the culture medium for calcified nodule formation have been published [26-28]. The presence of Dex is essential for the formation of hard tissue by stem cells, and Tf was identified as an essential growth factor for many cell types [29]. Tf is considered to be a natural and essential component for cell growth in tissue culture [30], and it has been frequently used in serum-free medium. In culture medium, the secondary role of Tf is to bind endogenous metal ions that can cause cytotoxicity [31]. This function of Tf may be important in cell culture. Metal chelating agents, such as transferrin, lactoferrin and ceruloplasmin, can function as antioxidant systems by binding harmful metal ions [32].

In this study, the proliferation of rBMCs in the medium containing Dex was hypothesized to be promoted by the addition of Tf. The effects of Tf on rBMCs were examined, but no increased proliferation/differentiation or hard tissue-forming ability of rBMCs was observed. ALP activity of rBMCs after the subculture with Dex was not significantly different from that of those in medium supplemented with Dex and Tf. Furthermore, in the subculture with Tf and Dex, quantitative analysis of DNA did not demonstrate a significant increase in cell number. This suggests that Tf does not increase the proliferation of stem cells in rBMCs *in vitro*. The addition of Tf to the medium resulted in a significant increase in the amount of Ca^{2+}, suggesting that differentiation from stem cells to hard tissue-forming cells was promoted by Tf. Tf may affect the growth of osteoblasts. Therefore, in this *in vitro* examination in which stem cells were cultured in a medium supplemented with Dex as a hard tissue inducer, the addition of Tf was hypothesized to be effective.

A previous study demonstrated that Tf plays a role in hard tissue formation [6]. Ekblom and colleagues found that Tf is required for cell proliferation and differentiation using rat organ cultures of developing teeth [33]. Thus, Tf is an important factor for stem or blast cells to form hard tissue. Hard tissue formation by the combination of insulin-Tf-selenium was not examined in this study. Regarding the promotion of hard tissue formation, the effects of Tf with Dex in this study were not compared with those of the cocktail reported in prior studies. The experimental methods likely differ and their comparison was considered to be inappropriate. However, Tf was confirmed as a factor for hard tissue formation in this study.

rBMCs will differentiate into chondrogenic cells with a mixture of ascorbic acid, insulin-transferrin-selenate and transforming growth factor-β1 in the medium [3]. On the other hand, bone formation is induced by Dex, β-glycerophosphate and ascorbic acid, which are well-known differentiation inducers [34, 35]. The amount of Ca^{2+} from the calcified nodules after the culture of rBMCs in this study was significantly increased by the addition of Tf and Dex to the medium. Thus, calcified nodule formation was promoted by the effects of Tf and Dex. The amount of calcified aggregates formed by the addition of Dex was significantly increased by the addition of Tf. This study demonstrated that Tf further promotes osteogenesis when added to the medium with Dex. Therefore, Tf should be recommended as an important co-factor for bone formation.

5. Conclusion

Tf is a chondrogenic stem cell differentiation inducer in addition to ascorbic acid, insulin, selenate and transforming growth factor-β1. However, there are no reports on the combined effects of Tf and Dex. Therefore, the formation of calcified nodules by cultured rBMCs was examined in medium containing Dex and Tf in this study. However, the effects of a mixture of insulin, transferrin, selenate and transforming growth factor-β1 should be compared with those of Tf and Dex observed in this study in the future.

Calcified nodule formation was promoted by the combined effects of Tf with Dex in the culture medium of rBMCs in this study. There was a significant increase in the amount of Ca^{2+} from calcified nodules by rBMCs cultured with Tf and Dex in the medium. This study confirmed significant effects of Tf on the promotion of bone formation. Therefore, the combined use of Tf and Dex may improve bone formation *in vitro*.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare no conflicts of interest associated with this manuscript.

Statement of ethical approval

Regarding the use and care of the animals, the Animal Welfare Committee of Osaka Dental University approved. The experimental procedures of this research also approved ethically by the Committee.

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