Enhanced proliferation and differentiation of osteoblasts induced by co-culturing with Schwann cells

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Abstract

Introduction: Transplanting Schwann cells into implant sockets might be an effective method to promote sensory responses of osseointegrated implants as a result of the well-known properties of Schwann cells in nerve regeneration. However, it is still unclear whether or not Schwann cells would disturb the osteogenic function of osteoblasts.
Material and methods: Using Transwell inserts, osteoblasts derived from neonatal rat calvariae were co-cultured with Schwann cells, and assessed via methylthiazol tetrazolium (MTT) colorimetric assay, Alizarin red staining, alkaline phosphatase (ALP) measurement and osteocalcin (OCN) quantification. The expression of osteogenic marker genes ALP, OCN, and collagen type I (COL-1) was evaluated by quantitative real time polymerase chain reaction (PCR) on day 3, 6 and 9.
Results: Compared with the control, there was a significant increase in the proliferation of co-cultured osteoblasts on day 3 and 6 ($P<0.05$), whereas no difference was shown on day 9. Co-culture of these two types of cells also led to a significant increase in ALP activity and OCN secretion on day 6 and 9. An elevated number of calcified nodules was observed on day 21. In addition, gene expression of ALP, OCN and COL-1 was highly upregulated by Schwann cells.
Conclusions: These findings demonstrated that the proliferation and differentiation of osteoblasts could be enhanced by co-culturing with Schwann cells.

Key words: osteoblasts, Schwann cells, co-culture, proliferation, differentiation.

Introduction

Osseointegrated dental implants have been widely used and are regarded as a useful and reliable treatment for the replacement of missing teeth. Because the periodontal ligament is lacking around osseointegrated implants, however, the Ruffini endings of the periodontal ligament can no longer signal information about mechanical events. Studies on implant loading have shown that the sensory thresholds of an implant are 10-100 times higher than those of natural teeth [1-3]. Without the self-protective mechanism afforded by the periodontal ligament, excessive forces may be loaded on the implant, and may result in damage to peri-implant bone [4]. Therefore, we previously intended to improve the sensory responses of osseointegrated dental implants with a method involving use of a Schwann cell graft [5].

Schwann cells are the glial cells of the peripheral nerve system, and play indispensable roles during neural development and regeneration [6, 7]. In periodontal ligament, Schwann cells extend their cytoplasmic processes

toward the axon terminals of Ruffini endings and wrap themselves around them. Some bioactive molecules produced by Schwann cells make an important contribution to the maturation and regeneration of periodontal Ruffini endings [8, 9]. When a Schwann cell graft is applied to repair peripheral nerve injuries, it has been reported to have a preferential effect on functional recovery [10, 11]. Therefore, a similar performance might be anticipated when Schwann cells are transplanted into implant sockets.

To verify our hypothesis, we previously studied cytocompatibility between Schwann cells and titanium implants. The results demonstrated that Schwann cells could grow well on various titanium implant surfaces and maintain their properties in nerve regeneration [12, 13]. However, another prerequisite that must be clarified is whether Schwann cells will interfere with osseointegration around dental implants. We have been unable to identify any report examining the effect of Schwann cells on osteogenic functions of osteoblasts, which play a crucial role during the initial stage of osseointegration. The purpose of the present study was to investigate the effect of Schwann cells on the proliferation and differentiation of osteoblasts.

Material and methods

Primary culture of Schwann cells

Primary Schwann cells were obtained by methods similar to those first described by Brockes et al. [14]. Briefly, the bilateral sciatic nerves of neonatal Sprague-Dawley rat pups (day 2) were dissected and washed three times with phosphate-buffered saline (PBS, pH 7.4). The epineurium of each nerve was stripped under the stereomicroscope. Nerves were cut into pieces (<1 mm) and enzymatically dissociated using 0.1% type I collagenase (Sigma, St. Louis, Mo.) for 30 min and 0.25% trypsin (Sigma, St. Louis, Mo.) for 10 min at 37°C. The resulting cell suspension was centrifuged (1,000 rpm, 8 min), placed into 25 mm^2 culture flasks (Costar Corning) and incubated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (penicillin-streptomycin solution). The antimitotic agent cytosine arabinoside (10^{-5} M) was added to the culture medium 24 hours after initial plating and incubated for 2-3 days to remove fibroblasts. Finally, Schwann cells were confirmed with the S-100 protein staining technique. The number of S-100 positive and negative cells was counted for 20 randomly selected fields under a microscope to calculate the purity of the cultured Schwann cells, which was found to be more than 93%.

Primary culture of calvarial osteoblasts

Osteoblastic cells were enzymatically isolated from calvariae of the same rat pups where Schwann cells had been harvested by the method described preciously [15]. Briefly, dissected calvariae were sequentially digested for 10 min intervals with 0.1% type I collagenase (Sigma) and 0.25% trypsin (Sigma) at 37°C. Cells obtained from the second to the fourth digestions were incubated in DMEM (Gibco) with antibiotics and 10% fetal bovine serum (FBS, Gibco). At about 90% confluence, cells were trypsinized and subcultured. To confirm that cultures were osteoblast enriched, mineralized bone nodule formation assay and alkaline phosphatase (ALP) staining were performed (data not shown). All experiments were performed using the cells of third or fourth passage.

Co-culture of Schwann cells and osteoblasts

To establish the co-culture system, 2×10^5 osteoblasts were seeded in six-well tissue culture plates, while 2×10^5 (Group A, see Table I) or 1×10^5 (Group B) Schwann cells were passaged on six-well Transwell clear polyester membrane inserts with 0.4-µm pore size (Costar Corning). After one day, Schwann cell inserts were transferred into the wells containing osteoblasts, so that two different cell types shared the culture medium but were not in physical contact. Nutrient media (DMEM with 10% FBS and antibiotics) were changed every three days. As a control, osteoblasts were cultured under the same conditions without Schwann cells.

Assessment of cell proliferation

Cell proliferation was assessed using a methylthiazol tetrazolium (MTT, Sigma, St. Louis, MO.) method. Briefly, 1 ml of serum-free medium and 100 µl of MTT solution (5 mg/ml) were added to each sample, followed by incubation for 4 hours at 37°C for MTT formazan formation. The medium and MTT were then replaced by 1 ml of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. After 30 min, 200 µl of supernatant was transferred to microplate wells (Falcon), and the optical density was quantified against a DMSO solution blank in a microplate reader at the wavelength of 492 nm (HTS 7000p1us, PerkinElmer, USA).

Measurement of ALP activity

On day 3, 6 and 9, ALP activity was determined in cell homogenates prepared by ultrasonication, with an ALP diagnostic reagent of p-nitrophenyl phosphate (Merit Choice Co. Ltd. Beijing, China). The absorbance of p-nitrophenol formed by the hydrolysis of p-nitrophenyl phosphate, catalyzed by ALP, was measured at 405 nm by a microplate reader (HTS 7000 p1us, PerkinElmer, USA). Total protein content was measured with the Bradford method, read at 595 nm and calculated according to bovine gamma globulin standards. ALP activity was expressed as unit/g protein.

Table I. Co-culture groups

Group code	Schwann cells	Osteoblasts
A	2×10^5	2×10^5
B	1×10^5	2×10^5
Control	N/A*	2×10^5

**N/A – not applicable*

Enzyme-linked immunosorbent assay (ELISA)

To quantify the concentration of osteocalcin (OCN) in cell cultured supernatant, commercially available ELISA kits were used strictly according to the manufacturer's instructions (Bionewtrans Pharmaceutical Biotechnology Co. Ltd. Franklin, MA.). Constitutive OCN secretion was analyzed following 3, 6 and 9 days of co-culture. Prior to analysis, the supernatant was centrifuged to remove cell debris.

Alizarin red staining

The ability of osteoblasts to form calcified bone nodules was assessed using Alizarin red staining technique. After co-culture with Schwann cells for 21 days, osteoblasts were washed, fixed in 4% formaldehyde, incubated for 10 min at room temperature in 1% Alizarin red solution (pH 4.2), and then rinsed with PBS.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from each sample by the TRIZOL method strictly following the manufacturer's protocol (Invitrogen, Carlsbad, CA.). The concentration and purity of freshly isolated RNA were measured at 260 nm using a spectrophotometer and with A260/280 ratio respectively. The first-strand cDNA was synthesized from 1 µg of RNA with murine leukaemia virus reverse transcriptase (Takara, Japan), and used for quantitative real-time PCR. Expression levels of representative genes, including ALP, OCN and collagen type I (COL-1), were quantified with an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, USA) and SYBR green PCR reaction mix (Infinigen, USA). Primers designed in primer express 2.0 analysis software and the annealing temperature for each gene are listed in Table II. The program used was 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and annealing temperature for 1 min. Melting analysis and agarose gel electrophoresis were performed to confirm the specificity of the PCR products obtained using each primer pair. The relative expression levels of genes were analyzed using the $2^{-\Delta\Delta Ct}$ method [16] by normalizing with β-actin housekeeping gene expression, and presented as fold increase relative to the control group.

Statistical analysis

All experiments presented here were run three times with triplicate samples. Data were expressed as means ± SD, and statistically analysed using one-way ANOVA followed by Newman-Keuls post hoc tests. Differences at probability of less than 0.05 were considered statistically significant.

Results

Proliferation of osteoblasts

As shown in Figure 1, co-culture of Schwann cells stimulated the proliferation of rat primary osteoblasts as measured by MTT assay. In the early period, both groups A and B had significantly higher levels of osteoblast viability than that of the control ($P<0.05$, see Figure 1). On day 6, the MTT OD values of group A and B increased to 0.816±0.037 and 0.740±0.042 respectively, about 56 and 41% higher than that of the control (0.524±0.086) ($P<0.05$). After 9 days of co-culture, cells of all three groups reached high confluence, and no significant difference was observed.

ALP activity and OCN secretion

As to the ALP activity, an indicator of calcification in the matrix maturation phase, the presence of Schwann cells did indeed increase the ALP activity of osteoblasts during the observation period ($P<0.05$) (Figure 2A). Meanwhile, the OCN concentration

Table II. Specific primers designed following the cDNA sequences of each gene in GenBank

Gene	GenBank accession number	Primers	Product size [bp]	Annealing temperature [°C]
ALP	NM_013059	(+), 5'-CGTCTCCATGGTGGATTATGCT-3'	209	61.5
		(–), 5'-CCCAGGCACAGTGGTCAAG-3'		
OCN	NM_013414	(+), 5'-TACCTCAACAATGGACTTGGAGC-3'	206	67.0
		(–), 5'-CTGGAGAGTAGCCAAAGCTGAAG-3'		
COL-1	XM_213440	(+), 5'-GCATGGCCAAGAAGACATCC-3'	239	60.0
		(–), 5'-CTCCTATGACTTCTGCGTCTGGT-3'		
β-actin	NM_031144	(+), 5'-CACGGCATTGTCACCAACTG -3'	167	
		(–), 5'-TTGAAGGTCTCAAACATGATCTGG -3'		

of both group A (4.98±0.45 ng/ml) and group B (4.89±0.62 ng/ml) was much higher than that of the control group on day 6 ($P<0.05$) (Figure 2B). On day 9, the same situation was observed and group A reached the highest level of 5.31±0.29 ng/ml.

Bone nodule formation

As shown in Figure 3, on day 21, after staining with Alizarin red, discrete mineralized bone nodules could be visualized by the naked eye as red-purple spots. Compared with the control, both co-cultured groups had an increased number of calcified nodules and more intense red staining.

Expression of osteogenic genes

Using real-time quantitative PCR, we noted that expression of osteogenic genes was upregulated by co-culturing with Schwann cells. For example, expression of ALP mRNA was improved over 5-fold on day 6 for both groups A and B (Figure 4A). OCN, a marker of terminal osteogenic differentiation, demonstrated obvious increased mRNA expression (Figure 4B). On day 9, over 8-fold upregulation of this gene was seen in group A, which was much higher than the upregulation in group B (less than 3-fold that of the control, $P<0.05$). COL-1 gene expression was upregulated in both group A and group B during the entire observation period (Figure 4C). Moreover, the gene expression in group B (4-, 5-, and 4.5-fold on day 3, 6, and 9 respectively) was increased to a much greater degree than that in group A (3.5-, 2- and 1.5-fold on day 3, 6, and 9 respectively) (Figure 4C).

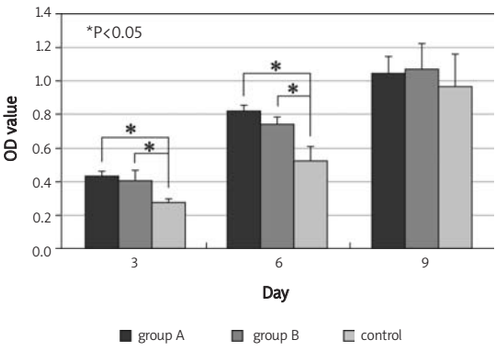

Figure 1. Proliferation of co-cultured osteoblasts measured by MTT assay. On day 3 and 6, both group A and B had significantly higher levels of osteoblast viability than that of the control. After 6 days of co-culture, the MTT OD values of group A and B were about 56 and 41% higher than that of the control respectively

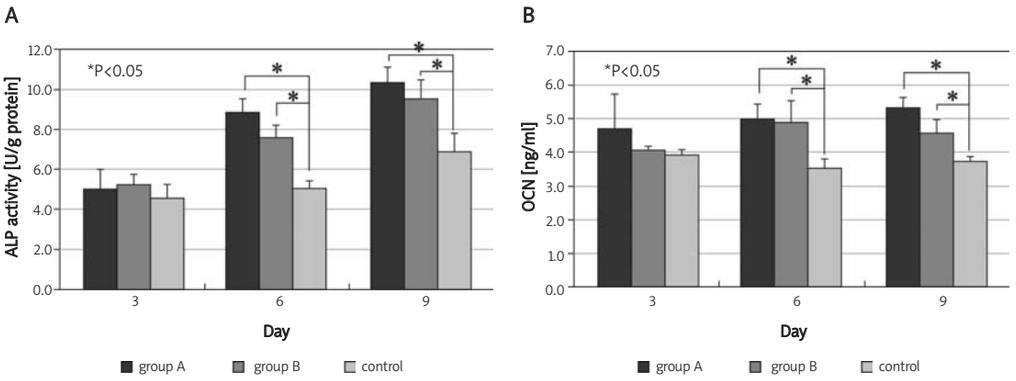

Figure 2. The effect of co-culture on ALP activity and OCN secretion was assessed on day 3, 6 and 9. **A** – significant increase of ALP activity was observed on day 6 and 9. **B** – the secretion of OCN was much higher on day 6 and 9 in both groups A and B

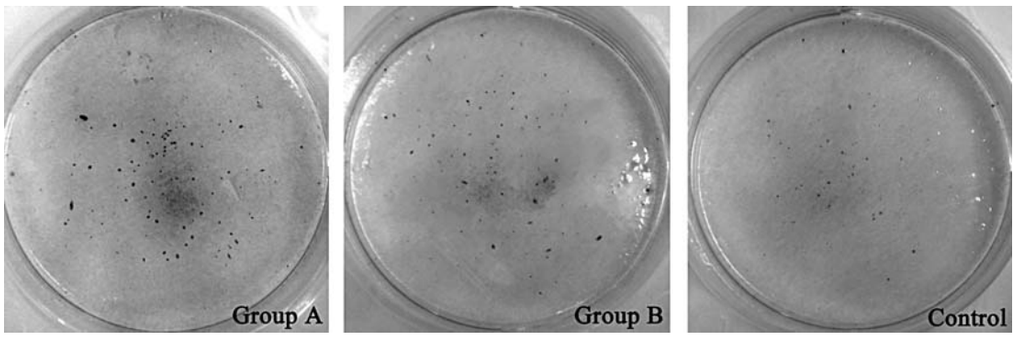

Figure 3. Alizarin red staining of osteoblasts. Compared with the control, co-cultured osteoblasts formed many more mineralized bone nodules after 21 days

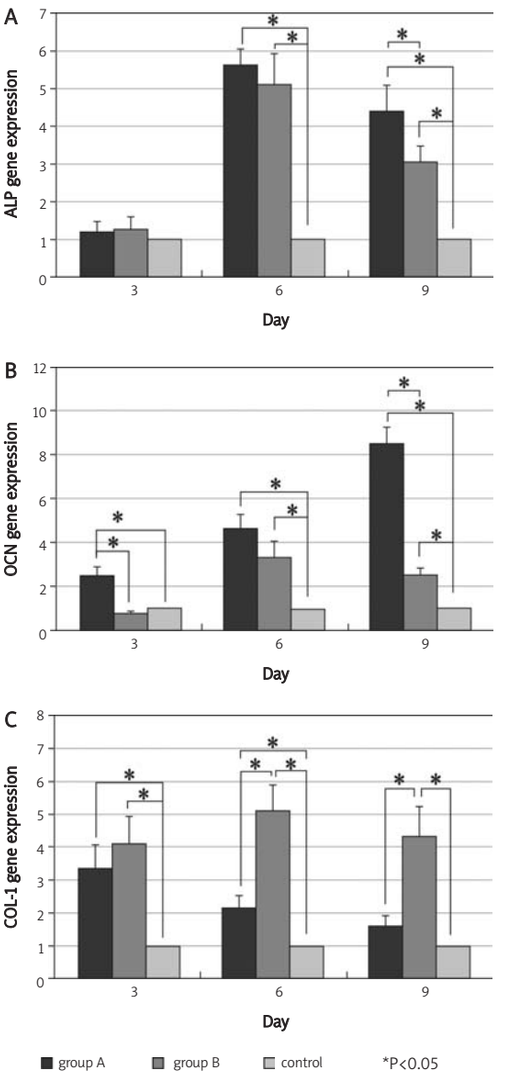

Figure 4. Expression of osteogenic-specific markers by osteoblasts co-cultured with Schwann cells. **A** – the level of ALP mRNA expression was improved over 5-fold on day 6 for both group A and B. **B** – OCN, a marker of terminal osteogenic differentiation, demonstrated obviously higher mRNA expression. **C** – the level of COL-1 was significantly greater than that of the control, especially for group B

Discussion

Since Branemark proposed the theory of osseointegration, it has been recognized that osseointegration is fundamental for the success of dental implants [17, 18]. During the initial process of osseointegration, osteoblasts are mainly responsible for the new bone formation around dental implants. In this study, to test whether Schwann cells would interfere with the osseointegration or not, the effect of Schwann cells on the proliferation and differentiation of osteoblasts was evaluated using a co-culture system. It was demonstrated that osteoblasts co-cultured with Schwann cells could proliferate more rapidly and secrete more OCN than osteoblasts cultured alone. Furthermore, co-cultured osteoblasts expressed greater levels of differentiation markers (for example, 5.5-fold more ALP, 4-fold more OCN, and 2-fold more COL-1 for group A on day 6) than single cultured osteoblasts. The capacity for bone nodule formation in osteoblast populations, which indicates the most advanced stage of osteogenic differentiation *in vitro* [19], was also evaluated in this study. Compared with the control, co-cultured osteoblasts had formed many more mineralized bone nodules by day 21. These findings indicated that Schwann cells would not disturb the functions of osteoblasts, but would, in fact, enhance their proliferation and differentiation.

Since increased proliferation and differentiation of primary rat calvarial osteoblasts in the presence of Schwann cells was observed without any contact between these two different types of cells, it suggests that osteoblasts may respond to certain soluble factors probably secreted by Schwann cells. It is well known that a variety of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are synthesized and released by Schwann cells [6, 7, 20]. These factors may bind to their receptors located on the cell membrane, and then exert wide biological effects on not only sympathetic and sensory neurons, but also various types of non-neuronal cells [21-24]. They could change the adhesion, proliferation, or gene profile of tumour cells, epithelial cells, dental pulp cells, and so on. The presence of trkA, trkB and trkC, which are the receptors of NGF, BDNF, and NT-3 respectively, has also been reported in osteoblastic cells [25-27].

NGF, one of the most important neurotrophic factors, is known to play an important role in the survival of osteoblastic cells [28]. Yada et al. [29] found that NGF might promote ALP activity and collagen biosynthesis of osteoblastic MC3T3-E1 cells. Recently, outstanding stimulation of proliferation, ALP and COL-1 mRNA expression by NGF in human pulp cells was also reported [24]. In this study, a similar increase in ALP activity was observed, and the mRNA expression of ALP, OCN and COL-1 was also obviously upregulated. Moreover, OCN secretion was greatly increased. These findings may indicate that Schwann cells enhance the differentiation of primary osteoblasts through an NGF pathway.

In this study, increased MTT OD values were observed in the co-culture system on day 3 and 6. Iwata et al. [30] noted that exogenous NT-3 could induce DNA-binding activities of particular transcription factors in osteoblastic cell lines. These transcription factors may modulate transcription of certain genes, which are related to proliferation of

osteoblastic cells. In addition, trkC, the receptor of NT-3, was highly expressed in the exponential growth phase of the osteoblastic cell line and decreased as the cells reached the differentiation stage. This may indirectly explain the positive effect of Schwann cells on the proliferation of primary osteoblasts.

In this study, two different concentrations of Schwann cells (group A, 2×10^5; group B, 1×10^5) were seeded in Transwell inserts and co-cultured with osteoblasts. Since group A showed a constant tendency of higher MTT values, ALP activity, OCN secretion and mRNA expression of ALP and OCN, it might indicate that more Schwann cells have a better performance on enhancing the proliferation and differentiation of osteoblasts. However, the COL-1 mRNA level in group B was higher than that in group A. This suggests that the effect of Schwann cells on osteoblasts may be dose-dependent. Mizuno et al. reported that neurotrophins at the concentration of 25 and 100 ng/ml could stimulate the mRNA expression of ALP and COL-1 in dental pulp cells [24].

In conlusion, the findings observed in this study suggest that Schwann cells could positively affect the proliferation, differentiation and bone nodule formation of osteoblasts. This effect may be caused by neurotrophic factors secreted by Schwann cells. These findings, together with the results we reported previously [12, 13], provide information to support the hypothesis that a Schwann cell graft may be a potential method of promoting sensory responses of osseointegrated implants [5]. Further animal experiments are needed to elucidate the effect of a Schwann cell graft on osseoperception and osseointegration *in vivo*.

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