1. INTRODUCTION

Brain and neural clinical hospitality have been rapidly advancing, including implantation techniques. Otherwise discreditable accidents sometimes happened. It is necessary to study efficiency and safety of techniques and materials for brain and neural cell proliferation and development. Precise mechanisms by which neurogenesis and gliogenesis are regulated in the central nervous system (CNS) remain to be elucidated. Telencephalic neuroepithelial cells contain neural precursors that give rise to the neuronal lineage and the glial lineage, which includes astrocytes and oliogodendrocytes (1, 2). The fate of neural precursors in the developing brain is believed to be determined by intrinsic cellular programs and by external cues, including implantation of biomaterials and cytokines (3). Doetsch et al. demonstrated that subventricular zone (SVZ) astrocytes act as neural stem cells in both the normal and regenerating brain (4). Neural stem cells, endogenously present in spinal cord *in vivo*, proliferate in response to injury, yet the vast majority of newly generate cells are glial fibrillary acidic protein (GFAP)-positive astrocytes (5). In addition, adult hippocampus-derived neural stem cells, when implanted into adult brain in such a region as cerebellum or striatum, have been reported to differentiate predominantly into glial cells (2, 6, 7).

Biodegradable polymers have been attractive candidates for scaffolding materials because they degrade and the new tissues are formed, although adverse events such as foreign-body reaction, inflammation and tumor formation were reported in clinical human and animal study. These scaffolds have shown great promise in the research of engineering a variety of tissues. Biodegradable polymer poly (L-lactic acid) (PLLA) is frequently implanted in cranial surgery etc. However, to engineer clinically useful tissues and organs is still a challenge. The understanding of the principles of scaffolding is far from satisfactory, still more its effect and safety on neural tissues are not known. We previously reported PLLA suppressed proliferation and differentiation of fetal rat midbrain neural precursor cells (8). In this report, we investigated the effect of PLLA on normal human astrocytes (NHA).

2. MATERIALS AND METHODS

Astrocyte cell culture

We used normal human astrocyte (Cambrex Bio Science, Walkersville, MD). NHA were seeded into 12-well plates for quantitative RT-PCR at a density of 2 x 10^4/well, or 24-well plates for MTT assay at a density of 1 x 10^4/well in ABM medium(Cambrex Bio

Science) supplemented with 5% FCS, rhEGF and IGF, and cultured in a humidified atmosphere of 5% CO_2 in 95% air at 37°C.

PLLA preparation

Stock solutions of PLLA were made in dimethyl sulfoxide (DMSO) and final concentration of DMSO was 0.1%; this concentration did not affect proliferation and development of NHA. Control cultures were incubated with 0.1% DMSO. Stock solutions of lactic acid and tin chloride were made directly in ABM medium.

MTT assay

After cell culturing for 1 week with PLLA, the viability of NHA cells was determined by MTT assay. The TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan) was used to measure changes of cell numbers. This assay is a nonradioactive alternative to tritium-thymidine incorporation. The system measures the conversion of tetrazolium salt compound into a soluble formazan product by the mitochondria of living cells. NHA in 24-well plates were cultured as described above. One week after NHA cultured with vehicle or PLLA, the media were replaced with 300 μl of fresh medium containing 6 μl TetraColor ONE reagent. After 2h, samples were measured in a micro plate reader.

Expression of neural cell marker genes

Total RNA was prepared from NHA using a modified acid guanidium thiocyanate-phenol-chloroform method. The total RNA treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) were subjected to reverse transcription using oligo d(T) primer (Toyobo, Tokyo, Japan) and superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42°C for 30 min followed by RNase H treatment. Aliquots of the cDNA (1/20) were used as templates for PCR analysis using Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 μl mixture including 1 μl of RT reaction, 2 μl Light Cycler-Fast Start Reaction Mix SYBR Green 1(Roche, Mannheim, Germany), 0.5 μM/liter of each primer, and 3 mmol/liter $MgCl_2$. The PCR program consisted of 40 cycles of 8 sec at 94°C, 5 sec at 65°C, 10 sec at 72°C. Primer sequences for amplification are 5'- CTAAGGAGGAGATTGGACAGG-3' and 5'-AGTGGTGGCAGTGATTT CAGT-3' for Nurr-1 amplification, 5'-TCCGCTGCTCGCCGCTCCTAC-3'and 5'-TCATCTCTGCCCGCTCACTGG -3' for GFAP amplification, 5'-TCGCCCTGCCCACTTGACTTC-3' and 5'-TTCCACACCTCCACGCTC TGA-3' for Id-3 amplification, 5'-GAGATCAGAGCCCAGGATGCT-3' and 5'-CTGAGGGGTGGTGCCAAGGAG -3' for Nestin amplification, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-

TCCACCACCCTGTTGCTGT A-3' for GAPDH. RNA preparation and RT-PCR in the present study were performed in triplicate.

Statistical analysis

The Fisher's PLSD was used to compare the PLLA concentration and relative expression levels of neural specific marker mRNA.

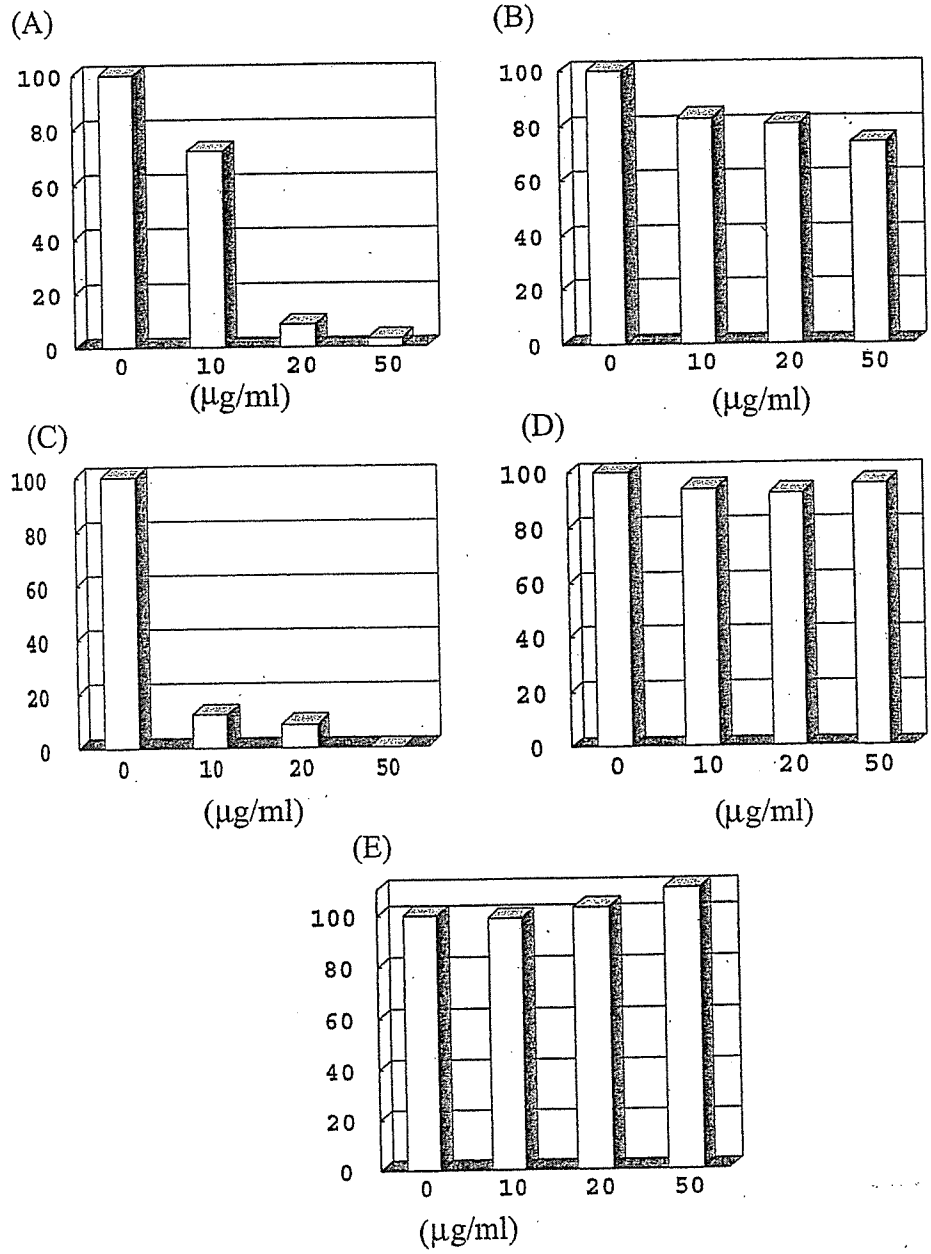

Fig. 1 Effect of PLLA on NHA proliferation (A) PLLA 3.000 (B) PLLA 5.000 (C) PLLA 11.000 (D) Lactic acid (E) Tin chloride

3. RESULTS AND DISCUSSION

NHA proliferation

We used three kinds of PLLA. PLLA 3000 (PLLA, Mw 3000) is made without catalyst. PLLA 6000 (PLLA, Mw 5000) is made with organic tin catalyst. PLLA 11000 (PLLA, Mw 11000) is made with catalyst tin chloride, contains 590 ppm tin. After a week culture with PLLA, we detected cell number of NHA using MTT assay. Cell numbers were decreased in a dose-dependent manner of PLLA (Fig. 1A-C). The cell number of NHA cultured with 50 μg/ml of PLLA 3000, PLLA 5000 and PLLA 11000 were 15%, 70% and 7.8% of that of control respectively.

Whether tin ion included in PLLA affected NHA proliferation or not, we added tin chloride to NHA culture medium (Fig. 1D). The concentration of tin chloride at 50 ng/ml did not affect NHA proliferation. PLLA is hydrolysed in medium, we assayed lactic acid (LA), a monomer of PLLA was also tested by the MTT assay using NHA cells. (Fig. 1E). There was no effect on the cell number of NHA culture with LA monomer. The cause of PLLA effect for NHA was neither included tin ion nor degraded LA monomer. It was probably the effect of PLLA itself and/or degraded LA oligomers.

Lam and his co-workers demonstrated that predegraded PLLA (P-PLLA; 25 kGy gamma-irradiation) caused signs of cell damage, cell death, and cell lysis due to phagocytosis of a large amount of P-PLLA particles (9). Phagocytosis of LA oligomers or degraded PLLA particles may affect the proliferation and development of NHA. It is necessary to know culture medium with PLLA contains how much PLLA particles, PLLA oligomer and organic tin.

Gene expression of neural cell specific markers

It has been suggested that a part of astrocytes contain neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte itself. The recent discovery of stem cell populations in the CNS has generated intense interest, since the brain has long been regarded as incapable of regeneration (5, 10, 11). Neural stem cells (NSCs) have capability for expansion and differentiation into astrocytes, oligodendrocytes, and neurons in vitro (12, 13). NSCs have been suggested to have therapeutic potential for central nervous system regeneration (14-16).

They express their original specific genes, neural cell specific markers. Neural precursor cells express Nestin, a class IV intermediate filament protein. Differentiated neuron expresses Nurr-1, a transcription factor and Id-3, a transcription inhibitory factor. Astrocyte expresses

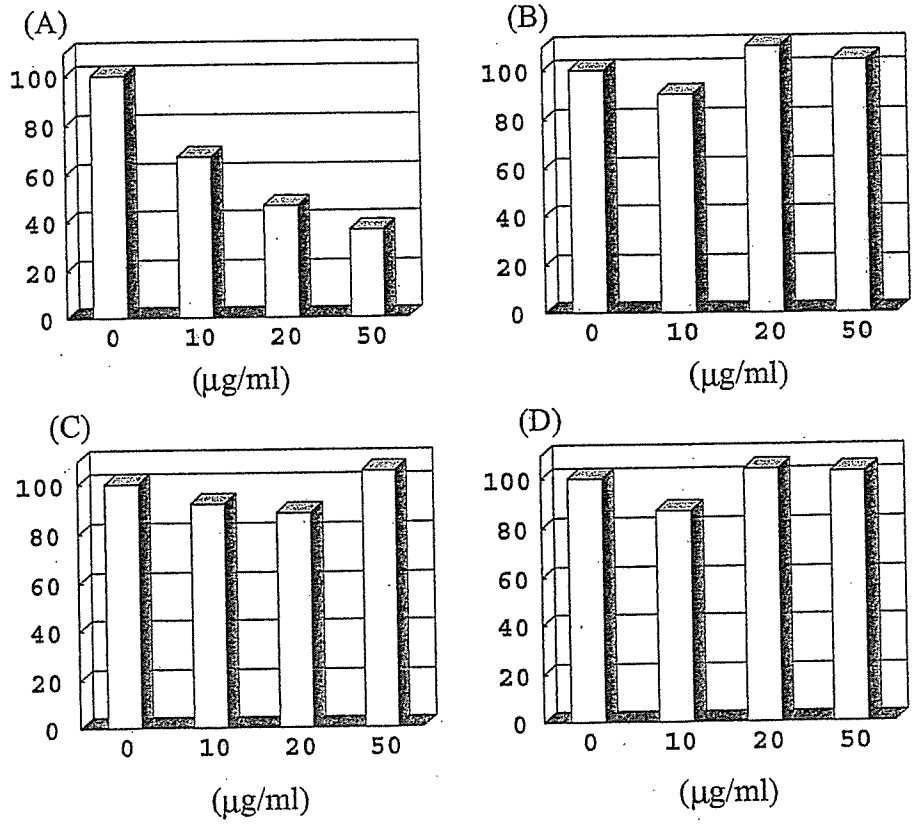

Fig. 2 Effect of PLLA on neural specific gene expression.
(A) Nestin (B) Nurr-1 (C) GFAP (D) Id-3

GFAP, a glial filamentous acidic protein. We compared gene expression of neural cell specific markers. Expression of Nestin, a neural precursor cell marker decreased with the dose of PLLA5000. The expression of Nestin in NHA cultured with 50 μg/ml PLLA was 30% of control (Fig. 2A). Expressions of the other genes that assayed in this study were similar to control (Fig. 2B-D).

Expression of Nestin was decreased when NHA were cultured with PLLA suggested that PLLA decreased population of neural precursor cells. There were two kinds of possibilities. (1) PLLA leads NHA to gliogenesis. Nakashima et al. reported that Gliogenesis significantly reduced the number of cells expressing Nestin and the number of cells expressing microtubule-associated protein 2 (MAP2), a neuronal marker. (2) When neural precursor cells specifically phagocytosed PLLA, they go to programmed cell death, apoptosis or loose their developmental potential as neural precursor cells. Lam et al. demonstrated that PLLA caused signs of cell damage, cell death, and cell lysis due to phagocytosis of a large amount of P-PLLA particles. Phagocytosis of PLLA may affect proliferation and development of NHA.

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Regulation of Chondrocyte Differentiation Level via Co-culture with Osteoblasts

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ABSTRACT

The close apposition of osteoblasts and chondrocytes in bone and their interaction during bone development and regeneration suggest that they may each regulate the other's growth and differentiation. In these studies, osteoblasts and chondrocytes were co-cultured *in vitro*, with both direct and indirect contact. Proliferation of the co-cultured chondrocytes was enhanced using soluble factors produced from the osteoblasts, and the differentiation level of the osteoblasts influenced the differentiation level of the chondrocytes. In addition, the chondrocytes regulated differentiation of the co-cultured osteoblasts using soluble factors and direct contact. These data support the possibility of direct, reciprocal instructive interactions between chondrocytes and osteoblasts in a variety of normal processes and further suggest that it may be necessary to account for this signaling in the regeneration of complex tissues comprising cartilage and mineralized tissue.

INTRODUCTION

REGENERATING TISSUES COMPRISING MULTIPLE CELL TYPES with specific and complex organization is a major goal of tissue-engineering studies.[1] There are still numerous issues to be clarified before these types of tissues can be regenerated, including understanding the interaction between the different types of cells, controlling signaling cascades that regulate cell proliferation and differentiation, and regulating the spatial distribution of the various cell types. It has recently been reported that several tissues composed of multiple different types of cells can be regenerated by transplanting the various cell populations together on polymer scaffolds.[2–4] For example, the co-transplantation of rat calvarial osteoblasts (RCOs) and bovine articular chondrocytes (BACs) on an appropriate carrier can lead to the formation of a growth plate–like tissue in severe combined immunodeficient (SCID) mouse.[4] The RCO and BAC were randomly distributed inside the gels initially but reorganized to form clearly separated bony and cartilage-like tissues. The cartilage area of the tissue was composed of normal and hypertrophic chondrocytes, and the spatial localization of the different types of chondrocytes correlated to the location of the mineralized tissue. This finding suggested that there were interactions between the RCOs and BACs that induced hypertrophic differentiation of the BACs.

The growth plate is a highly organized structure composed of chondrocytes at different stages of differentiation between the epiphyseal and metaphyseal bone at the distal ends of the bone. Various growth factors and hormones, including fibroblast growth factors (FGFs), transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), and parathyroid hormone-related protein (PTHrP), regulate the growth and structure of the growth plate.[5] BMPs have chondro-inductive effects on mesenchymal stem cells, and FGFs and insulin-like growth factor (IGF) have been reported to regulate chondrocyte proliferation, whereas TGF-β and PTHrP inhibit chondrocyte maturation.[5–8] BMP is also known to induce osteogenesis, and TGF-β and FGFs

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are also recognized as important regulators of bone development and growth.[9–13] We hypothesized that growth factors secreted by each cell type could regulate the formation of the growth plate–like tissue after implantation of gels containing RCOs and BACs. Because many of the relevant growth factors can be secreted from osteoblasts and chondrocytes, co-culture of osteoblasts and chondrocytes could potentially enhance chondrogenic differentiation as well as osteogenic differentiation. It has been reported that chondrocytes express soluble factors selectively promoting osteogenesis of mesenchymal stem cells.[14] However, the effects of co-culture of osteoblasts on chondrocyte differentiation have not been reported.

The effect of RCO and BAC co-culture *in vitro* on the differentiation level of each cell type was investigated in these studies. RCOs and BACs were cultured alone or mixed in 12-well plates, and their differentiation level was determined qualitatively and quantitatively. To more directly assess the effects of soluble factors produced from RCOs or BACs on the other cell type, a transwell system was also used to co-culture RCOs and BACs while avoiding their direct contact.

MATERIALS AND METHODS

Materials

Medium, fetal calf serum (FCS), antibiotics, and other supplements for cell culture were purchased from Invitrogen Co. (Carlsbad, CA). All chemicals were purchased from Sigma (St. Louis, MO) unless stated. All reagents and primers for performing real-time polymerase chain reaction (PCR) were obtained from Applied Biosystems (Foster City, CA).

Cell culture

Primary RCOs were isolated from 1- to 2-day-old newborn Lewis rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) and maintained in alpha-minimum essential medium (MEM) containing 10% FCS and 1% penicillin–streptomycin.[4] Primary BACs were isolated from freshly slaughtered calf forelimbs and maintained in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) containing 10% FCS and 1% penicillin–streptomycin.[4] The cells were maintained in incubators under standard conditions (37°C, 5% CO_2, 95% air, saturated humidity). The passage number of RCOs and BACs used in this study did not exceed 3 and 4, respectively. Both cells were used for differentiation studies when sub-confluent.

Co-culture study

The RCOs, the BACs, and a mixture (RCO:BAC = 2:1) were seeded in 12-well plates at the total density of 9×10^4/2 mL medium/well using a 1:1 mix of alpha-MEM and DMEM/F12 containing 10% FCS (the area cell density was $2.4\times10^4/cm^2$). Some studies were performed using the medium containing 10 mM beta-glycerophosphate, ascorbic acid (50 μg/mL), and 10^{-8} M dexamethasone to determine the effects of RCO differentiation on BACs in the co-culture. Cells were maintained in incubators under standard conditions. In all assays, culture media were changed once a week, and the cell numbers and differentiation levels assessed every 2 weeks. To determine whether the effect of RCOs and BACs on each other depended on direct cell–cell contact, a Transwell system (Corning Costar Co., Cambridge, MA) was also used for the co-culture experiments. RCOs and BACs were separately seeded into the wells or inserts and incubated for 4 weeks in those studies. The pore size and the area of an insert of the Transwell were 0.4 μm and 1.1 cm^2, respectively. The total cell number and the ratio of RCO and BAC seeded were kept similar to the simple co-culture system described above (9×10^4/2 mL medium/total well, RCO:BAC = 2:1). Therefore, the area densities of RCOs and BACs in the bottom well were 1.6×10^4 and $0.8\times10^4/cm^2$, respectively. The area cell density in the insert well was adjusted to $2.7\times10^4/cm^2$ when RCOs were cultured or to $5.4\times10^4/cm^2$ when BACs were cultured in the bottom of the Transwell system.

The total cell number in each test well was determined using a Coulter counter (Coulter Electronics Inc., Hialeah, FL) after collecting cells. The cells were washed using phosphate-buffered saline (PBS) and detached by adding 0.5 mL of 0.05% trypsin solution and 0.5 mL of collagenase solution (5 mg/mL, F. Hoffmann-La Roche Ltd, Basel, Switzerland). After detachment, the cell number in the suspension was determined.

Differentiation levels of RCOs were estimated from changes in their alkaline phosphatase (ALP) activity, calcium deposition, and osteocalcin production. Total ALP activity of RCOs was estimated using the original procedure of Ohyama *et al.*[15] Briefly, RCOs or co-culture were washed using PBS (–), followed by addition of 1 mL of 0.1 M glycine buffer (pH 10.5) containing 10 mM magnesium chloride, 0.1 mM zinc chloride, and 4 mM p-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the added buffer at 405 nm was detected using a microplate reader to evaluate ALP activity. Calcium deposition during the test period was qualitatively estimated using alizarin red staining.[16] To determine osteocalcin production from the RCOs, their culture medium was replaced with fresh medium without FCS 1 day before the desired sampling, and the new medium was collected after 24 h. The total amounts of osteocalcin in this medium were estimated using an osteocalcin enzyme-linked immunosorbent assay kit (Biomedical Technologies Inc., Stoughton, MA).

To determine effects of co-culture on the BAC differentiation level, Alcian blue staining was applied as a qualitative method to estimate production and deposition of proteoglycans and glycosaminoglycans from BACs.[17] In

TABLE 1. SEQUENCES OF PRIMERS FOR DETECTION OF BOVINE MESSENGER RIBONUCLEIC ACID EXPRESSION BY REAL-TIME POLYMERASE CHAIN REACTION

GAPDH	Forward	TCCCCACTCCCAACGTGT
	Reverse	ATCTCATCATACTTGGCAGGTTTCT
Type II collagen	Forward	GCATTGCCTACCTGGACGAA
	Reverse	CGTTGGAGCCCTGGATGA
Type X collagen	Forward	CCTCTTTCTCAGGATTCTTGGTG
	Reverse	TTCAAGCAGATTTGTGTTAGCTCG
Aggrecan	Forward	GAGTGGAACGATGTCCCATGT
	Reverse	GCATTGATCTCGTATCGGTCC

some cases, proteoglycan-bound dye was extracted overnight with 4 M guanidine hydrochloride at 4°C, and the quantity determined from absorbance at 600 nm.[18,19] For quantitative analysis, a real-time PCR technique was applied. Briefly, total ribonucleic acid (RNA) from BACs was collected using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA). After its reverse transcription, real-time PCR was performed to detect expression of aggrecan, type-II and type-X collagen messenger RNA (mRNA) using ABI Prism 7700 (Applied Biosystems). To normalize the data, mRNA expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also determined. Sequences of primers for the mRNA are shown in Table 1.

Gap junctional intercellular communication (GJIC) between cells was analyzed using fluorescene-activated cell sorting (FACS) according to Dürig *et al.*[20] Briefly, one cell type, stained with calcein, was seeded in a culture dish containing the other cell type that had already been stained with the PKH 26 fluorescent dye. After incubation, all cells were trypsinized to prepare a cell suspension and subjected to FACS analysis to determine the ratio of exhibiting fluorescence from both markers (PKH 26 and calcein) as an estimate of the GJIC level between the 2 cell types.

STATISTICS

All data were expressed as mean ± standard deviation. The Fisher-Tukey criterion was used to control for multiple

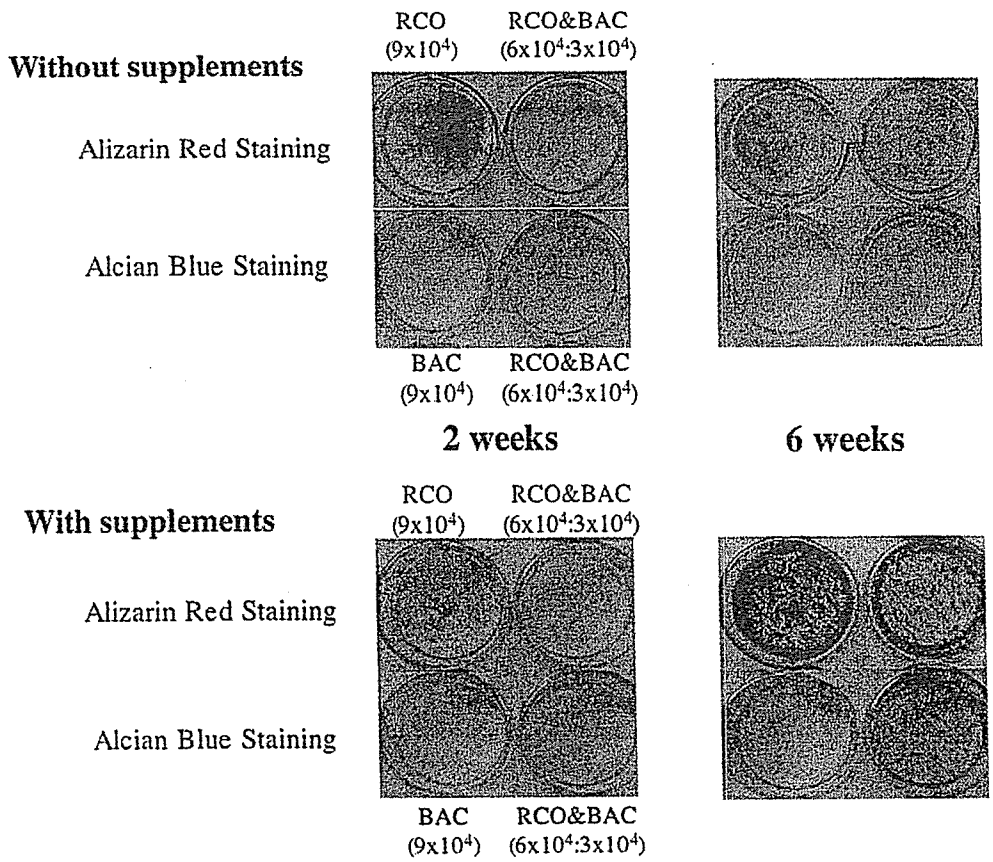


FIG. 1. Change in differentiation level of rat calvarial osteoblasts (RCOs) and bovine articular chondrocytes (BACs) by their coculture in alpha-minimum essential medium/Dulbecco's modified Eagle medium/F12 (1:1) containing 10% fetal calf serum with or without osteogenic supplements [dexamethasone (10 nM), beta-glycerophosphate (10 mM), and ascorbic acid (50 μg/ml)], which enhance osteoblast differentiation. Calcium deposited by RCOs and proteoglycan produced by BACs were qualitatively estimated using alizarin red staining and Alcian blue staining, respectively. Results from a single culture of each type at the same time point are shown in the left well. The number of each type on day 0 is indicated in the figure.

comparisons and to compute the least significant difference between means.

RESULTS

Direct co-culture studies

RCOs and BACs were first mixed in a ratio of 2:1 and co-cultured in 12-well plates for 6 weeks. The effect of co-culture was qualitatively analyzed using alizarin red and Alcian blue staining (Fig. 1). Alcian blue staining indicated that co-culture led to greater proteoglycan and glycosaminoglycan deposition after 2-week culture, even though the number of BACs on the starting day in co-culture was one third of the control group containing BACs only. These effects were maintained over time. The total cell number of RCO-alone culture, BAC-alone culture, and co-culture after 6-week culture were approximately 7.7×10^5, 15.8×10^5, and 15.1×10^5, respectively. Similar to results obtained in the absence of osteogenic supplements, co-culture with osteogenic reagents again enhanced BAC differentiation. In contrast, no enhancement of RCO differentiation with co-culture was observed using alizarin red staining. Addition of osteogenic supplements enhanced RCO differentiation in solo culture, but again co-culture appeared to have no effect on the intensity of alizarin red staining. The total cell number of RCO-alone culture, BAC-alone culture, and co-culture after 6-week culture with the supplements was approximately 1.0×10^6, 1.1×10^6, and 1.7×10^6, respectively.

The level of osteogenesis was next quantitatively analyzed for RCOs in co-culture and solo culture in the presence of osteogenic supplements. Figure 2 (A and B) shows the ALP activity and the production rate of osteocalcin, without normalization by cell number, in RCO solo culture

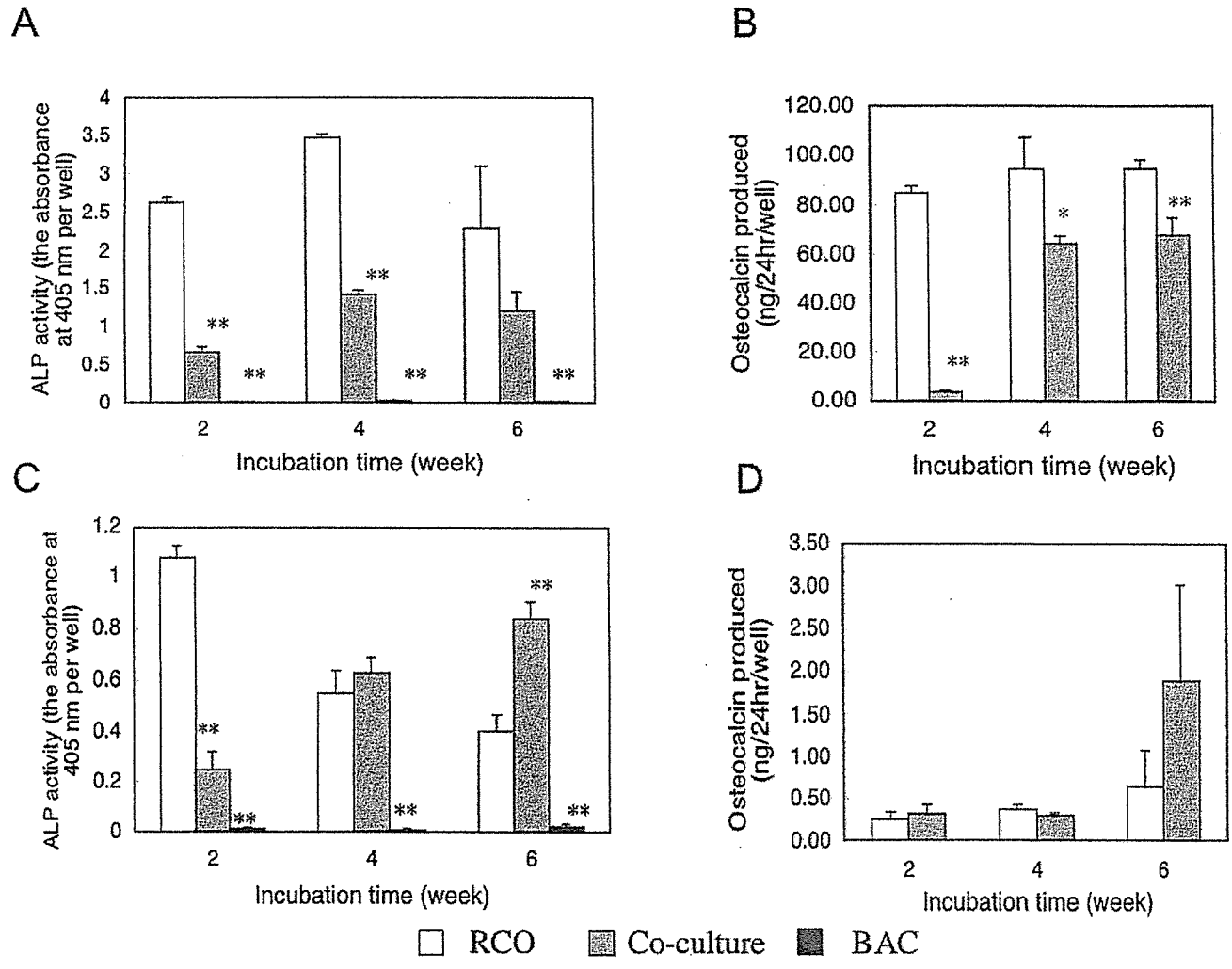


FIG. 2. Effect of co-culture with bovine articular chondrocytes (BACs) on rat calvarial osteoblast (RCO) differentiation, estimated from alkaline phosphatase activity expressed as the absorbance at 450 nm (**A, C**) and the amount of osteocalcin secretion into the medium (**B, D**). RCOs (open column), BACs (closed column), and RCOs with BACs (gray column) were cultured in medium containing 10% fetal calf serum with the osteogenic supplements (**A, B**) or without the supplements (**C, D**). Data not normalized by the cell number, $*p<0.05$, $**p<0.01$ against respective single RCO culture.

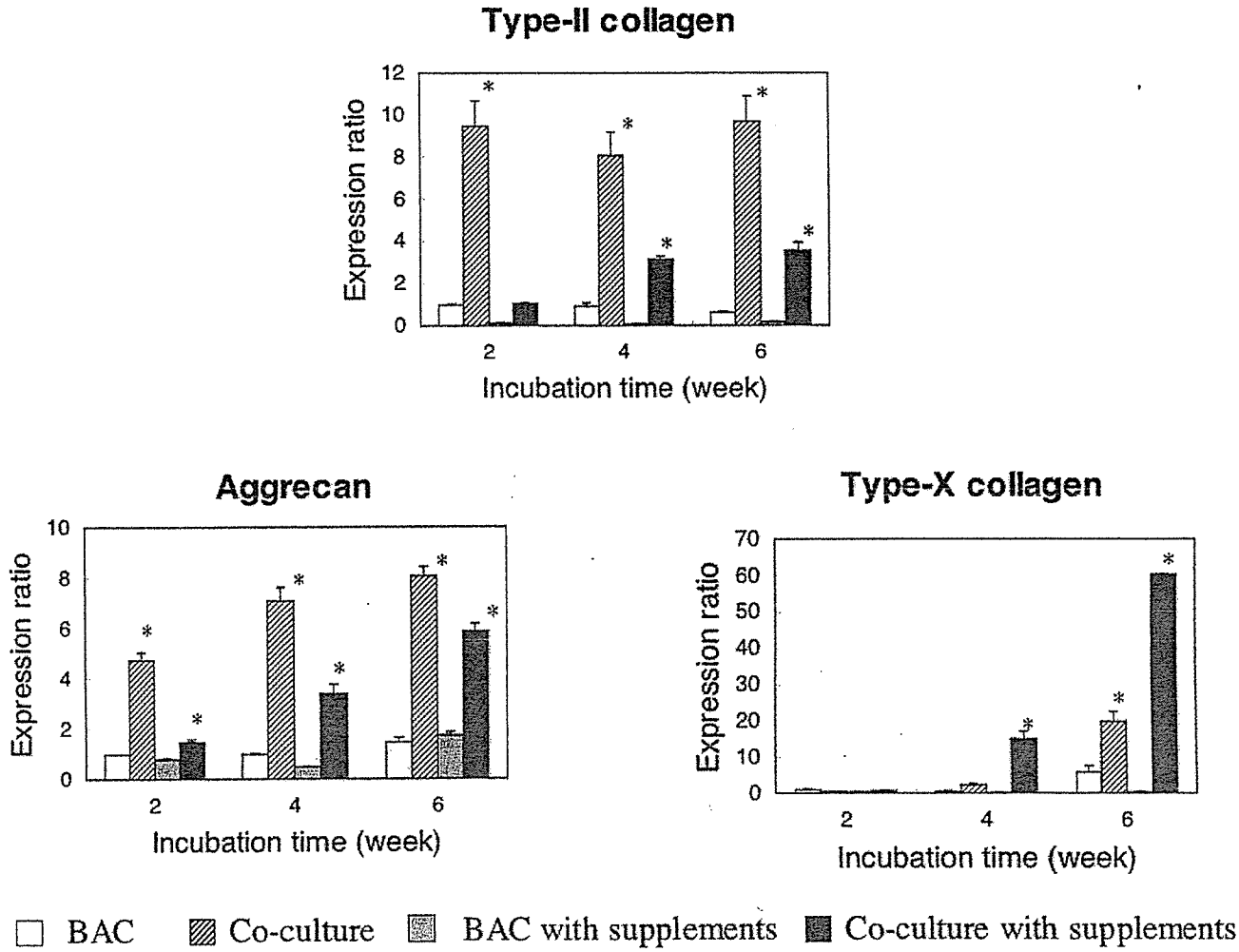


FIG. 3. Change in expression level of type-II collagen, aggrecan, and type-X collagen messenger ribonucleic acid (mRNA), which indicates the differentiation level of chondrocytes, during co-culture of bovine articular chondrocytes (BACs) with rat calvarial osteoblasts (RCOs). Cells were cultured in medium with or without the osteogenic supplements (open column: BACs alone without the supplements; striped column: co-cultured BACs without the supplements; gray column: BACs alone with the supplements; closed column: co-cultured BACs with the supplements). All data were normalized using the expression level of glyseraldehyde-3-phosphate dehydrogenase mRNA and expressed as a ratio of the expression level in the BAC-alone culture at 2 weeks without the osteogenic supplements (*$p < 0.01$ against BAC-alone culture under the same condition).

and co-culture with BACs with osteogenic supplements. The ALP activity detected in the RCO-alone culture was always greater than that in co-culture system, irrespective of incubation time (Fig. 2A). The magnitude of these effects appears reasonable, because the RCO number in the co-culture is likely to be less than that in RCO-alone culture, as based on initial seeding densities. No measurable ALP activity was noted in BAC-alone cultures. Similar to the ALP activity, osteocalcin production from the co-culture system was less than with RCO-alone cultures. However, the amount of osteocalcin production in the co-culture condition markedly and significantly increased after 4 weeks (Fig. 2B, $p < 0.01$ against co-culture group at 2 weeks).

Quite different results on RCO differentiation were noted when these analyses were performed on cells cultured without osteogenic supplements. ALP activity in the co-culture condition increased over time, whereas ALP decreased over time in RCO-alone culture, and ALP activities were significantly greater in co-culture at the later time points (Fig. 2C). Again, minimal ALP activity was noted in BAC-alone cultures. The osteocalcin production from RCOs in co-culture or single culture increased with time but was low even after a 6-week incubation (Fig. 2D). Co-culture of RCOs with BACs led to a 3-fold higher osteocalcin secretion rate at the last time point, although this difference was not significant (Fig. 2D).

Total RNA from the co-culture was also extracted, and the expression of mRNA for type-II and type-X collagen and aggrecan was analyzed with or without osteogenic supplements. When the cells were cultured without the osteogenic supplements, mRNA for type-II collagen and

TABLE 2. EFFECT OF 4-WEEK CO-CULTURE OF RAT CALVARIAL OSTEOBLASTS (RCOs) AND BOVINE ARTICULAR CHONDROCYTES (BACs) ON CELL NUMBER AND ALKALINE PHOSPHATASE ACTIVITY OF RCOs USING TRANSWELL CULTURE SYSTEM TO AVOID THEIR DIRECT CONTACT

Culture condition		*Cell number*		*ALP activity (per 1×10^5 cells)* *(the absorbance normalized by RCO number)*	
Position	*Cell type*	*With osteogenic supplements*		*With osteogenic supplements*	
Well	RCO	$5.55\times10^5\pm6.2\times10^3$	$7.21\times10^5\pm2.9\times10^4$	0.056 ± 0.003	0.349 ± 0.013
Insert	RCO	$2.40\times10^5\pm8.2\times10^3$ **	$2.64\times10^5\pm2.3\times10^4$	0.109 ± 0.004 **	0.531 ± 0.040
Well	RCO	$7.30\times10^5\pm3.4\times10^4$	$8.00\times10^5\pm8.5\times10^4$	0.035 ± 0.003	0.381 ± 0.034
Insert	BAC	$5.25\times10^5\pm4.3\times10^4$	$8.97\times10^5\pm2.3\times10^4$ **	Not detected	Not detected
Well	BAC	$6.63\times10^5\pm4.0\times10^4$ **	$1.12\times10^6\pm8.9\times10^4$	Not detected	Not detected
Insert	RCO	$3.90\times10^5\pm1.3\times10^5$ *	$2.05\times10^5\pm5.7\times10^4$ **	0.065 ± 0.014	0.462 ± 0.113
Well	BAC	$5.66\times10^5\pm6.6\times10^3$	$1.02\times10^6\pm4.3\times10^4$	Not detected	Not detected
Insert	BAC	$2.71\times10^5\pm6.0\times10^3$	$6.14\times10^5\pm2.7\times10^4$	Not detected	Not detected

$^*p<0.05$, $^{**}p<0.01$.

aggrecan were markedly enhanced in co-culture with RCOs, even after only 2 weeks. In BAC-alone cultures, aggrecan and type-X collagen mRNA expression in BAC slightly increased after a 6-week incubation, whereas type-II collagen mRNA expression was maintained at a low level during the experimental period. A similar enhancement of BAC differentiation with co-culture was noted when cells were maintained in medium containing osteogenic supplements, although the magnitude of BAC differentiation, as measured according to type-II collagen and aggrecan expression, decreased (Fig. 3). Expression of type-X collagen mRNA was significantly enhanced after 4 to 6 weeks in this condition.

Additional studies indicated that there were direct GJICs between RCOs and BACs (data not shown), even though the 2 cell types were isolated from different species, suggesting that the RCOs and BACs might influence each other through this mode of communication.

Co-culture using transwell system

In transwell co-culture experiments that allow cells to share medium without direct contact, the number of RCOs and BACs increased more than with a single-cell culture after a 4-week incubation (Table 2). With osteogenic supplements, however, the increase in the number of co-cultured RCOs was not observed, and the increase in the number of co-cultured BACs was smaller than the increase in co-cultured BACs without the supplements. When cultures were stained using Alcian blue after a 4-week incubation, co-cultured BACs in the presence or absence of osteogenic supplements were stained more intensely than BAC-alone cultures under the same culture condition (Fig. 4A). Quantification of the Alcian blue dye extracted from cultures demonstrated that more bound dye per BAC was always present in co-cultures, but addition of osteogenic supplements suppressed proteoglycan deposition (Fig. 4B). Co-culture in the absence of osteogenic supplements in the transwell system did not enhance RCO differentiation. The RCO ALP level in co-culture normalized by cell number was approximately two-thirds that in RCO-alone culture (Table 2). When the osteogenic supplements were added to the medium, however, the alizarin red stained co-cultured RCOs more intensely than the RCO-alone culture (Fig. 4A). Moreover, ALP activity in co-culture and RCO-alone culture was similar in this condition (Table 2). The same results were observed in these experiments regardless of which cell type was placed in the bottom well or insert of the transwell system (data not shown).

DISCUSSION

Because it was previously reported that a growth plate–like tissue was regenerated when arginine-glycine-aspartic acid (RGD)–modified alginate gel discs containing a simple mixture of RCOs and BACs (ratio of 2:1) were implanted into SCID mice for 26 weeks,[4] unknown interaction between RCOs and BACs might play an important role in this tissue regeneration. *In vitro* co-culture experiments in the current study indicated that co-culture of BACs with RCOs enhanced BAC proliferation and differentiation levels via direct contact with RCOs and paracrine effects of soluble factors secreted from the RCOs and that the phenotype of the co-cultured RCOs influences the level of this enhancement. In addition, BACs regulate co-cultured RCOs, although the effects are lower in magnitude.

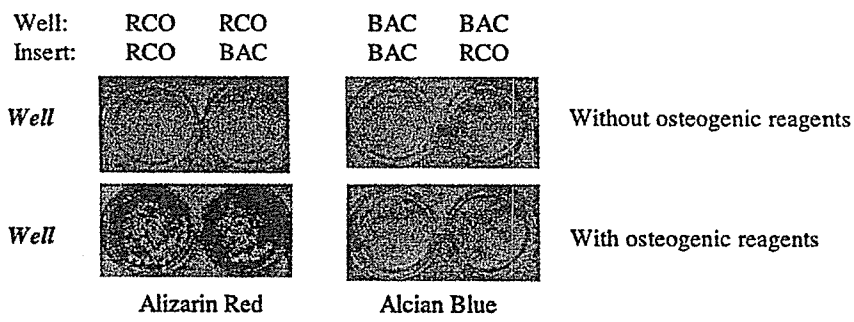
Simple, direct co-culture of RCOs and BACs increased the total cell number in the culture 1.5 to 2 times as much as RCO- or BAC-alone (only when osteogenic supplements were added) culture, although the contribution of each cell type to the total cell number was not assessed. However, transwell experiments (Fig. 4) suggested that the number of RCOs and BACs increased in co-culture. The mechanism of this effect must be clarified in future studies.

The results of this study indicated that not only cell proliferation but also cell differentiation was enhanced

using co-culture of chondrocytes and osteoblasts. BAC differentiation level was first estimated from Alcian blue staining (Fig. 1). Because Alcian blue molecules bind to proteoglycans,[17–19] the BAC differentiation level can be estimated from the density of Alcian blue binding to proteoglycans that are secreted from differentiated BACs. The magnitude of this effect in the co-cultures depended on whether osteogenic supplements were added to the medium, indicating that the differentiation level of the co-cultured RCOs influenced the BAC differentiation level. When mRNA levels of type-II collagen, aggrecan, and type-X collagen, which can be used as markers indicating chondrocyte differentiation,[21,22] were measured using real-time PCR, the expression of type-II collagen and aggrecan in BAC decreased with addition of the osteogenic supplements (Fig. 3). When BACs were co-cultured with RCO without the supplements, mRNA of type-II collagen and aggrecan were expressed 5 to 9 times as higher as those expressed in a single BAC culture. When these mRNA expression levels were quantified in BAC and RCO co-culture with the supplements, the levels were lower than the co-culture without the supplements. Alternatively, type-X collagen was strongly expressed in the co-culture with the supplements, and the level in this condition was 60 times as high as the single BAC culture and 3 times as high as the co-culture without the supplements. These findings suggest that co-culture of BACs with osteogenic supplements leads to differentiation into a hypertrophic phenotype[23] and indicate that the differentiation level of the co-cultured RCOs regulates the differentiation stage of the co-cultured BACs. The studies using transwell culture (Fig. 4) further suggested that soluble factors produced by RCOs are at least partially responsible for the enhanced BAC proliferation and differentiation level in co-culture, because there is no direct contact between RCOs and BACs in this condition. It has been previously demonstrated that many growth factors, including the TGFs, FGFs, and IGFs, play a role in chondrocyte differentiation.[5–8] One or two of these soluble factors may be secreted from RCOs to induce BAC differentiation. In addition, some components in FCS may have an additional effect on the BAC differentiation in concert with the cell-secreted soluble factors. The soluble factors that mediate these effects remain to be identified.

Enhancement of RCO differentiation was not observed with co-culture with BACs when analyzed using alizarin red staining (Fig. 1). Although one probable reason for this result was ascribed to the initially different RCO number in the co-culture, the ALP activity after 2-week culture was still lower than what would be expected based on the initial seeding density of the RCOs. The ALP activity was also suppressed in co-culture using the Transwell system even after 4-week culture (Table 2). This suggests the co-culture

A



B

Culture condition	The ratio of extracted alcian blue	
	Without osteogenic supplements	With supplements[†]
BAC-alone	1.000 ± 0.069	0.441 ± 0.141
BAC-RCO	2.999 ± 0.165**	1.144 ± 0.293*

FIG. 4. Effect of rat calvarial osteoblasts (RCOs) and bovine articular chondrocytes (BACs) co-culture on their differentiation using a transwell system to avoid direct contact between the 2 cell types. RCOs and BACs were separately cultured for 4 weeks with or without the osteogenic supplements. The differentiation level of RCOs and BACs in the bottom wells of the system were estimated using alizarin red and Alcian blue staining, respectively (**A**). To determine the differentiation level of BACs, proteoglycan-bound Alcian blue dye was extracted, and the absorbance of the extract was expressed as a ratio of the value obtained from BAC–BAC transwell culture without the osteogenic supplements. All data were normalized using BAC number (per 1×10^5 cells) (**B**) (*$p<0.05$, **$p<0.01$ against BAC–BAC culture in the same condition. $^{\dagger}p<0.01$ against the same co-culture combination without osteogenic supplements).

with BACs suppresses the differentiation of the RCOs in the early stages. However, the ALP activity of the cells in co-culture without the supplements increased as their incubation time increased, whereas the activity of single-RCO culture decreased over time. Because BACs appeared to differentiate to a hypertrophic phenotype with time (type-X collagen mRNA expression increased as incubation time increased), this ALP increase in the co-culture may be ascribed to an increase in ALP production from the hypertrophic chondrocytes. However, the osteocalcin production was increased after 6-week co-culture, although its value was still less than the values found in co-culture with the supplements (Fig. 2C, D). In addition, it has been suggested that hypertrophic chondrocytes regulate endochondral ossification.[23,24] Therefore, these findings suggest that co-culture may enhance some measures of *in vitro* RCO differentiation but not all. Expression of type-I collagen mRNA in solo culture and co-culture should be examined in the future to analyze the effect of co-culture on RCO differentiation in more detail, as well as to further analyze the differentiation level of the BACs. Alternatively, when ALP activity and osteocalcin production from the co-culture with osteogenic supplements were measured, both values were less than those from RCO-alone culture, irrespective of incubation time (Fig. 2A, B). These results suggest that maximal enhancement of RCO differentiation may be obtained with the supplements alone.

When results from simple co-culture of RCOs and BACs were compared with those from their co-culture using the transwell system, some inconsistencies between the results could be observed. For example, Figs. 1, 2A, and 2B suggest suppression in differentiation of RCOs co-cultured with BACs and osteogenic supplements, whereas results from transwell culture with supplements (Table 2 and Fig. 4) indicate no suppression in their differentiation. Similarly, BACs co-cultured with RCOs and the supplements in the transwell system has decreased deposition of proteoglycans, although simple co-culture with the supplements enhanced deposition of proteoglycans. These inconsistencies between direct and transwell co-culture could result from direct interactions (e.g., GJIC) that differentially regulate the phenotype of each cell type. The RCOs and BACs used in these studies were derived from different species, rat and bovine, and it was expected that there would be no direct interaction between these cell types. When GJIC, which is known to regulate homeostasis in cells and tissues[25–27] was estimated, communication between RCOs and BACs was observed (data not shown). Therefore, in addition to unidentified soluble factors, this communication and other direct interactions may play a role in controlling BAC and RCO differentiation in the co-culture system and probably in previous *in vivo* experiments. Clarifying these various types of cross talk between RCOs and BACs, as well as cross talk between these cells and stem cells in the future may be essential to clinically regenerating growth plate tissue effectively.

The results of this study indicate direct, reciprocal instructive interactions between co-cultured osteoblasts and chondrocytes. Therefore, it may be necessary to account for this signaling to regenerate complex tissues composed of several kinds of tissues and a mixture of various cell types, including stem cells. Similar studies using human cells will also be valuable to validate the importance of this work for human disease and regeneration.

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Enhancement of Differentiation and Homeostasis of Human Osteoblasts by Interaction with Hydroxyapatite in Microsphere Form

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Key words: osteoblasts, differentiation, homeostasis, hydroxyapatite, biocompatibility

Abstract. The aseptic loosening of artificial joints with associated periprosthetic bone resorption may be partly due to the suppression of osteoblast function to form new bone by wear debris derived from the joint. To assess the effect of wear debris on osteoblasts, we cultured normal human osteoblasts (NHOst) in contact with several kinds of microspheres as models of wear debris. The NHOst in contact with polystyrene, polyethylene, and alumina microspheres showed a lower differentiation level than NHOst alone as estimated from the amounts of deposited calcium. On the other hand, hydroxyapatite particles enhanced the differentiation of NHOst. In addition, sintered hydroxyapatite enhanced expression of osteocalcin mRNA and gap junctional communication of NHOst. This study suggests that polystyrene, polyethylene, and alumina microspheres have the potential to disorder not only the differentiation but also the homeostasis of NHOst in contact with them. However, hydroxyapatite enhanced the differentiation as well as the homeostasis of NHOst, even in microsphere form, suggesting its good biocompatibility as biomaterials for bone tissues.

Introduction

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, resulting in undesirable side effects. One good example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. Many researchers have reported that aseptic loosening with associated periprosthetic bone resorption is partly due to the activation of macrophages and osteoclasts by wear debris from the artificial joint [1-3], but few researches have focused on the interaction between wear debris and osteoblasts, especially normal human osteoblasts [4]. In this study, normal human osteoblasts were cultured in contact with various kinds of microspheres made from polymers or ceramics used as model wear debris, and the effects of the microspheres' characteristics and interaction conditions were discussed in regard to the proliferation, differentiation and homeostasis maintenance of the osteoblasts.

Materials and Methods

Microspheres. Monodispersed polystyrene (PS) microspheres with different diameters (0.1, 0.5, 1, 5, and 10 μm) were kindly supplied by Japan Synthetic Rubber Co., Ltd. (Tokyo, JAPAN). Low-density polyethylene (PE) microspheres were kindly provided by Sumitomo Seika chemicals Co., Ltd. (Tokyo, JAPAN). Alumina (Al_2O_3) microspheres were obtained from the Association of Powder Process Industry and Engineering. Sintered and un-sintered hydroxyapatite (HAp) microspheres (7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. (Chiba, Japan). Determined by Multisizer II (Coulter Electronics Inc., Hialeah, FL), the average diameters of PE and alumina microspheres were found to be 6.4 and 5.1 μm, respectively. Sterile microspheres and microsphere-coated plates were prepared by the method previously reported [5]. The obtained microspheres and microsphere-coated plates (20 μg/well) were subjected to the assays.

Cellular Assays. Normal human osteoblasts (NHOst) were purchased from BioWhitteker Inc. (Walkersville, MD). The cells were maintained using alpha minimum essential medium (Gibco) containing 20% fetal calf serum (FCS) in incubators (37°C, 5%-CO_2-95%-air, saturated humidity).

differentiated osteoblasts [15], were determined using the RT-PCR technique. Figure 2 shows time profiles of osteocalcin mRNA expression in NHOst cultured with pre-coated PS, PE, alumina, and two kinds of HAp microspheres. As shown in the figure, only the cells co-cultured with sintered HAp microspheres expressed osteocalcin mRNA after a 1-day incubation, while those co-cultured with other microspheres did not express the mRNA. This finding suggests that sintered HAp microspheres have the potential to induce osteocalcin production from NHOst. Neither spontaneous calcium deposition was observed by the incubation of sintered nor un-sintered HAp microspheres without NHOst, so that it is possible that the un-sintered HAp degrade in culture medium with NHOst, resulting in an increase of calcium concentration in the culture medium that enhances the calcium deposition by the NHOst. Therefore, it is suggested that sintered HAp can induce the differentiation of NHOst, and may be a suitable material for inducing osteogenesis rather than un-sintered one.

In conclusion, microspheres made from various materials had an effect on the differentiation of NHOst. The level of the effect varied with the size, amount, and composition of the microspheres. Microspheres made from PS, PE and alumina showed a potential to suppress the proliferation and the differentiation of co-cultured NHOst. On the other hand, microspheres made from HAp, especially sintered HAp, enhanced the differentiation of co-cultured NHOst, and showed their potential to maintain their homeostasis. Estimating the effect of various microspheres on the differentiation of osteoblasts will provide valuable information on the effects of wear debris from artificial hip joints as well as estimating their effects on osteoclast function.

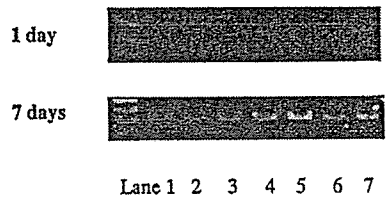

Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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diameter [5,9]. Taking into account our findings about the inhibitory effects of various microspheres on the functions of NHOst, it is probable that NHOst can phagocyte PS microspheres as well as macrophages, and in particular, may phagocyte microspheres 5 μm in diameter. Moreover, the effect of the added PS microspheres suggests that NHOst better recognize the microspheres from their lower than upper side. This may explain the reduced functions of NHOst co-cultured with the pre-coated 5 μm PS microspheres.

To estimate the effect of the material composing the microspheres, NHOst were cultured for 1 week on pre-coated PS, PE, alumina and HAp microspheres, all of which have a diameter of around 5 μm. Table 1 shows their number ratio and ALP activities, and the calcium amounts. Pre-coated PS, PE and alumina microspheres showed the potential to suppress functions of NHOst although some of these data did not show statistical differences against NHOst without microspheres. However, when NHOst were cultured with pre-coated HAp, the amount of calcium deposited was almost twice that detected in the cells without microspheres. It was observed that HAp microspheres have no potential to deposit calcium after a 1-week incubation without NHOst (data not shown). Therefore, the increase in calcium deposition by pre-coated HAp may be due to the enhancement in the differentiation of NHOst in contact with HAp. As expected, added various microspheres affected NHOst in a similar manner but less than the pre-coated microspheres (data not shown). We have hypothesized that GJIC of cells in contact with various biomaterials can be used as an index for estimating the biocompatibility of many kinds of biomaterials [5,6,9,11]. In addition, osteoblasts have been reported to communicate with one another *via* GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development [8,12]. Therefore, effects of these microspheres on the communication of co-cultured NHOst were estimated to consider the relation between this function and the differentiation of NHOst. The FRAP assay revealed that HAp microspheres enhanced the GJIC level of NHOst to 1.8 times as much as that of NHOst alone but others slightly inhibited it, indicating HAp has a potential to enhance homeostasis maintenance function of the NHOst as well as their differentiation. Details of the microspheres effects on GJIC of NHOst will be reported elsewhere [13]. These results indicated that the materials of microspheres affected the differentiation of co-cultured NHOst as well as the diameter of microspheres and their contact with the cells. In addition, microspheres made from HAp, which is a major component of bone tissue and has been shown to have good biocompatibility as bone substitute implants [14], may have the potential to enhance the differentiation of osteoblasts. These results suggest that the estimation of the effects of biomaterials in microsphere form on in vitro cell function may be useful for their in vivo biocompatibility evaluation.

We estimated the effect of sintering, normally used to harden HAp, on the function of NHOst. The estimation revealed that both HAp microspheres enhanced the amount of calcium deposited although the ALP activity of the cells decreased. In addition, when the un-sintered HAp microspheres were incubated with NHOst, the calcium deposition was observed more than sintered HAp. As another index of the differentiation of the NHOst, mRNA expression levels of osteocalcin, which is a well-known protein detected in

Table 1. Effects of a 1-week incubation with pre-coated microspheres on various functions of NHOst.
(Amounts of microspheres = 20 μg/well)

	Control	Polystyrene	Polyethylene	Alumina	Hydroxy Apatite (Sintered)
Diameter (μm)		5.0	6.4	5.1	7.2
The cell number ratio (%)	100.0±5.5	88.2±2.2	92.2±1.3	82.4±2.8	83.0±2.3
Percent ALP activity (activity/proliferation)	100.0±4.7	79.2±5.6	72.7±3.6*	58.2±5.7*	73.8±6.0*
Percent deposited calcium (Calcium percent/proliferation)	100.0±3.7	97.3±4.2	82.3±3.7	90.3±7.8	163.3±18.5*(a

Data are shown as the mean value ± SEM (n = 4 to 22)
* $p < 0.01$, against control group
(a) $p < 0.05$, against NHOst co-cultured with polyethylene and alumina microspheres

All assays were carried out using the medium supplemented with 10mM β-glycerophosphate. NHOst ($2x10^4$ cells/well/500 μl medium) were cultured on the microsphere-coated plates for estimating the effect of the microspheres from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst were cultured with microsphere-containing medium (20 μg/500 μl medium) after they had adhered to the collagen-coated plates. The cell number ratio of NHOst cultured with microspheres was evaluated using the alamar Blue™ assay (BioSource International, Inc., Camarillo, CA), which incorporates an oxidation-reduction indicator based on the detection of metabolic activity, according to manufacturer's instruction.

The level of alkaline phosphatase (ALP) activity of the NHOst and the amounts of calcium deposited during a 7-day incubation were evaluated to estimate differentiation level of NHOst as previously reported [6]. In addition, RT-PCR was performed to detect the expression of osteocalcin mRNA in the NHOst (primers for human osteocalcin [7]; forward 5'CATGAGAGCCCTCACA3' and reverse 5'AGAGCGACACCCTAGAC3'; product size 307-bp).

Gap junctional intercellular communication (GJIC), which is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules [8], among NHOst co-cultured with microspheres were evaluated using FRAP assay as previously reported [9].

All data were expressed as the mean value ± the standard deviation (SD) or the standard error of means (SEM) of the obtained data as indicated in all figures and tables. The Fisher-Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

Results and Discussion

Figure 1 shows the effect of the diameter of pre-coated polystyrene microspheres on proliferation, the ALP activity of co-cultured NHOst cells, and the amounts of deposited calcium on the NHOst. To compare the effect of the microspheres on the ALP activities and the calcium amounts for each NHOst, the obtained data were standardized based on the cell number ratio co-cultured with the microspheres. As shown in figure, suppression on ALP activity of NHOst and the amounts of deposited calcium were observed when 0.1 μm and 5 μm microspheres were co-cultured. When the microspheres were added after cell adhesion, they did not show a significant inhibitory effect on the functions of NHOst (data not shown). By pre-coating of the microspheres on the bottom of the test plates, the area they occupied became larger as their diameter became smaller. This increase in the microsphere occupied area would affect many functions of the test cells, resulting in the inhibitory effect of the 0.1μm microspheres on the function of NHOst when the same quantity of microspheres was coated. On the other hand, the suppression of ALP activity of NHOst and calcium deposition by pre-coated 5 μm PS microspheres suggests that not only the area they occupied but also their size may cause the unique inhibitory activity of the 5 μm PS microspheres. It is well known that the size of a microsphere plays an important role in phagocytosis [10], although it is unclear that there is the same size dependence on phagocytosis by the NHOst as by macrophages. In addition, our previous study suggested that even fibroblasts were likely to phagocyte microspheres of a specific

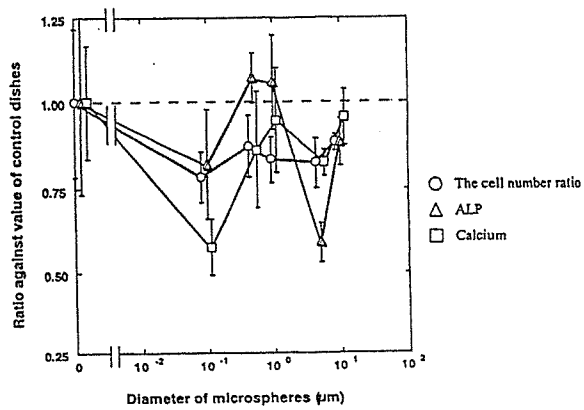

Figure 1. Effects of diameter of pre-coated PS on various functions of NHOst. Data are shown as the means±SD

SAFETY EVALUATION OF TISSUE ENGINEERED MEDICAL DEVICES USING NORMAL HUMAN MESENCHYMAL STEM CELLS

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Abstract: For safety evaluation of tissue engineered medical devices using normal human mesenchymal stem cells (hMSC), in this study, some genes expressions in hMSC were compared with those in two kinds of the tumor cells (HeLa and HepG2). Effects of the passage number of hMSC on the gene expressions were also investigated using quantitative real-time RT-PCR. The proliferation speed of hMSC was lowered with the cell passage number. The mRNA expressions of c-myc oncogene and nucleostemin in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC). And the mRNA expressions of them in hMSC decreased with the passage number. Wnt-8B mRNA was expressed in the tumor cells (HeLa and HepG2), but not in the stem cells (hMSC) in any passage number. Although these results suggest change in these expression levels are not directly related to the tumorigenesis of hMSC, it is discussed that mRNA expression levels of c-myc oncogene, nucleostemin, and Wnt-8B can be used as an index of hMSC tumorigenesis.

Key words: hMSC, tumorigenesis, c-myc, nucleostemin, Wnt-8

1. INTRODUCTION

Several recent studies demonstrate the potential of tissue engineering for regenerative therapy using somatic stem cells. Human mesenchymal stem cells (hMSC) derived from bone marrow aspirates have the potentiality to differentiate into osteocytes, chondrocytes, myocytes, stromal cells, tenocytes, adipocytes, and so on. Therefore, the autologous cell or tissue transplantation using hMSC was noticed as the medical treatment under the various kinds of clinical conditions. On the

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