concentration HA (1680 kDa)-coated dishes (0.01, 0.5, 1.0 mg/dish) already had attached and confluent but not in high concentration HA-coated dishes (2.0 mg/dish). These results indicated that the changes of cell viability by HA-treatment may depend on the cell attachment activity.

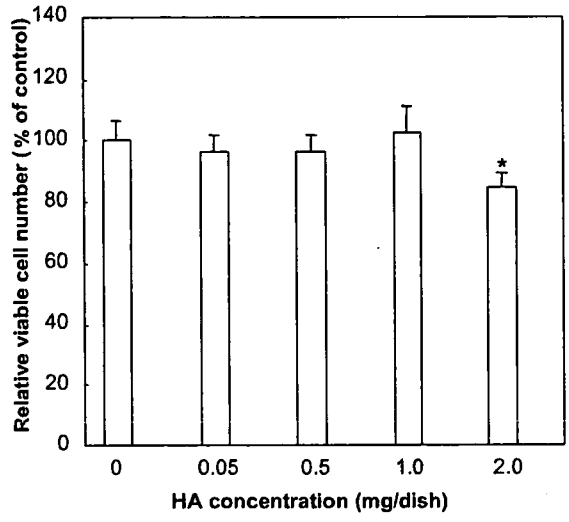

Figure 1. Viability of HIT-T15 cell after 72 h of HA-treatment. The viable cell numbers of HIT-T15 cell were determined by alamarBlue™ assay as described in Section 2. Each value denotes the mean ± S.D. $*P \leq 0.05$ compared to untreated control.

HIT-T15 cells, retain glucose-stimulated insulin secretion, showed a increase in insulin secretion as a function of stimulation. Thus, their insulin output was 13.25 ± 0.96 and 19.63 ± 0.98 pg/μg protein in the base and glucose-stimulation (11.1 mM), respectively (n = 9 dishes from three independent experiments) (data not shown). When these cells were exposed to low concentration of HA-coating (0.25, 0.5. 1.0 mg/dish), their insulin secretion was significantly increased in the absence or presence of glucose-stimulation. By contrast, high concentration of HA-coating (2.0 mg/dish) failed to increase its insulin secretion (Fig. 2).

On the other hand, when HIT-T15 cells were treated with HA-addition for 72 h, the increasing effect was not exhibited. The insulin secretion was without difference between control and HA-addition (data not shown). Previous studies have indicated that HA-treatment enhances the function of GJIC in normal human dermal fibroblasts [3] and the expression of Connexin43 in rat calvarial osteoblast [4]. The increasing evidence suggests that gap junction proteins and/or GJIC participate in the multifactorial control of insulin secretion. Thus, the increase in insulin secretion by HA-coating might have relation to gap junctions.

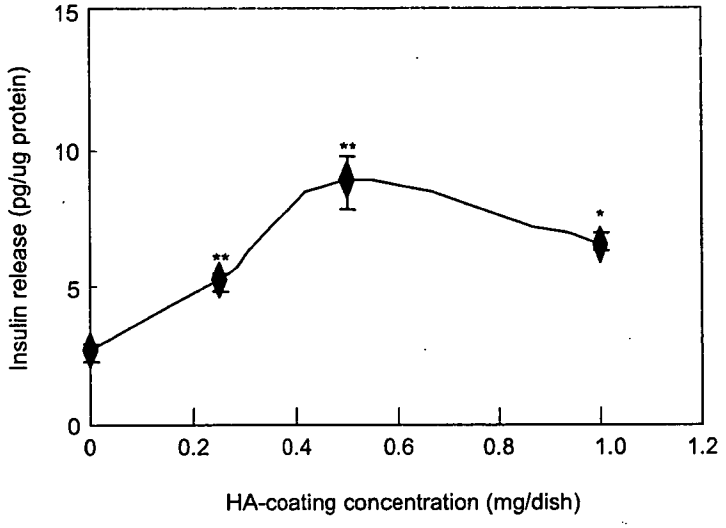

Figure 2. Concentration-dependent effects of HA-coating on insulin secretion from HIT-T15 cells. Treated with HA for 72 h, HIT-T15 cells were incubated with KRB buffer for 60 min. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean ± S.D. of three separate experiments. $*P \leq 0.05$, $**P \leq 0.01$ compared to control.

To test whether the HA-coating affects the gap junctions in pancreatic β-cells, we assessed the function of GJIC using Lucifer Yellow by SLDT assay. A scrape line was made on the cell grown to confluence, and the fluorescent dye penetrated the adjacent cells. The distance of dye transfer was determined.

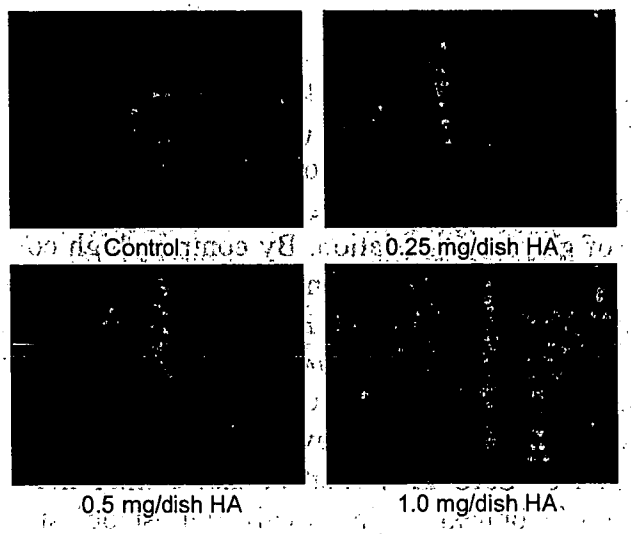

Figure 3. Time-course effects of various concentrations of HA-coating on the dye transfer ratio of HIT-T15 cells.

Fig. 3 shows the patterns of dye transfer in HIT-T15 cells treated with HA. The dye transfer extent of the cells grown on the HA-coated dishes

was more than that of the control, which indicated that GJIC function was activated by the HA-coating. The effect of HA is influenced by the concentration of FBS and the nutrients in medium, because the serum contains many components such as hormone, growth factor (FGF, etc.), cell adhesion molecule (N-CAM and cadherins), and transportation protein [4]. As a result, the HIT-T15 cells can use these nutrients and the nutrient-enriched substrata, e.g., natural extracellular matrixes with HA bound, to change the cell aggregations. Therefore, HA might play an important role in the increase of GJIC.

With the evidence above, it is known that the gap junction channels play a role in the regulation of β-cell secretion [5, 6]. It has been shown that the increase in connexin, e.g., gap junction proteins Cx43, affects the electrical coupling, synchronization of $[Ca^{2+}]_i$ oscillations, and insulin secretion, and the insulin secretion is evoked by a variety of metabolizable and nonmetabolizable secretagogues that activate different intracellular pathways [7-10]. In this study, we have found that the functional gap junction is promoted by low concentration HA-coating in spite of the inhibitory effects on the cell viability in high concentration HA-coating dishes. However, further intensive investigation should be promoted on the detailed action mechanism of HMW-HA responsible for the insulin-secreting activity.

4. CONCLUSIONS

The function of GJIC is considered to be a useful marker for evaluating tissue-engineered products. The data obtained in this study shows that gap junctions contribute to regulating some still-unknown mechanism to couple the stimulus-secretion of HIT-T15 cells under the condition of low concentration HA-coating. These results give useful information on how to design biomaterials of polysaccharides such as HA, when the GJIC is an important function for evaluating biocompatibility of biomaterials.

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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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other hand, owing to the similarity to the tumor cells, the stem cells also possess the ability of cell proliferation. Consequently, it is required to evaluate the safety of hMSC when that is used for tissue engineered medical devices. In this study, hMSC was compared with two kinds of the the tumor cells, HeLa (human cervix cancer) and HepG2 (human hepatoma), by investigating the differences in some genes expressions of each cells. Effects of the passage number of hMSC on the gene expressions were also investigated using quantitative real-time RT-PCR.

2. MATERIALS AND METHODS

Cell culture. Human mesenchymal stem cells (hMSC) purchased from the Cambrex Bio Science Walkersville, Inc. (MD, USA) was cultured in Mesenchymal Stem Cell Basal Medium (MSCBM; Cambrex Bio Science Walkersville, Inc.) supplemented with Mesenchymal Cell Growth Supplement (MCGS; Cambrex Bio Science Walkersville, Inc.), L-Glutamine and Pen/Strep at 37°C under a 5% CO_2 atmosphere. The cells was seeded at a density of 6,000 cells / cm^2 and subcultured when they are just sub-confluent (approximately 90% confluent) up to 10^{th} passage.

Quantitative RT-PCR. For quantitative RT-PCR, total RNA was extracted from hMSC of 1^{st}, 3^{rd}, 5^{th} and 10^{th} passage cultures with ISOGEN (NIPPON GENE CO., LTD.). RNA was then reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics; Tokyo, Japan). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, and Wnt-8B are summarized in Table 1. Amplifications of them were carried out for 10s at 95°C, for 15s at each annealing temperature, and for 12s at 72°C for 40 cycles. PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in Roche Light Cycler (software version 4.0).

Table 1. Primers and annealing temperatures used for Real time RT-PCR

Gene name	GenBank™ accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing Temp (°c)
c-myc	V00568	Forward	5'- GCG AAC ACA CAA CGT C -3'	1626	315	50
		Reverse	5'- CAA GTT CAT AGG TGA TTG CT -3'	1940		
nucleostemin	X91940	Forward	5'- CCA TTC GGG TTG GAG TAA -3'	782	284	50
		Reverse	5'- CTG TCG AGC ATC AGC C -3'	1065		
Wnt-8B	NM_014366	Forward	5'- AGT GAC AAT GTG GGC T -3'	331	244	60
		Reverse	5'- CGT GGT ACT TCT CCT TCA G -3'	574		

3. RESULTS

In this study, for safety evaluation of tissue engineered medical devices using normal human mesenchymal stem cells (hMSC), some genes expressions in hMSC were compared with those in two kinds of the tumor cells (HeLa and HepG2). At first, effect of the passage number on hMSC proliferlation was investigated. The proliferation speed of hMSC was lowered with the cell passage number (Fig. 1).

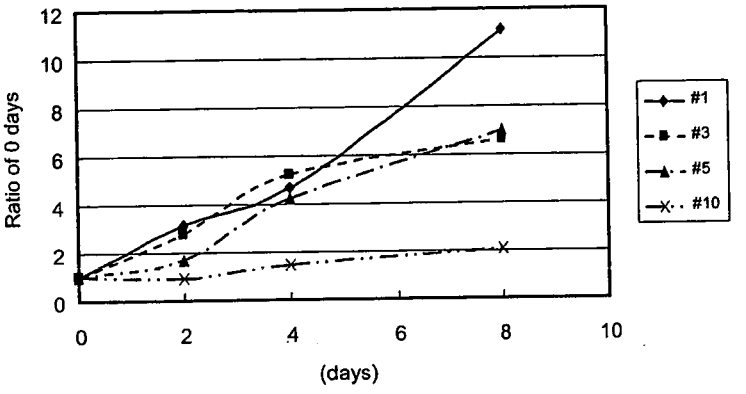

Fig. 1. Effect of the passage number on the cell growth curves of hMSC.

The mRNA expressions of c-myc oncogene in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC) (Fig. 2). The mRNA expressions of c-myc oncogene in hMSC in the 3rd and 5th passages were higher than in the 1st and 10th passages (Fig. 3). Similarly to c-myc, the mRNA expressions of nucleostemin in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC) (Fig. 4). The mRNA expressions of nucleostemin in hMSC decreased with the passage number (Fig. 5). Wnt-8B mRNA was expressed in the tumor cells (HeLa and HepG2), but not in the stem cells (hMSC) in any passage number (Fig. 6).

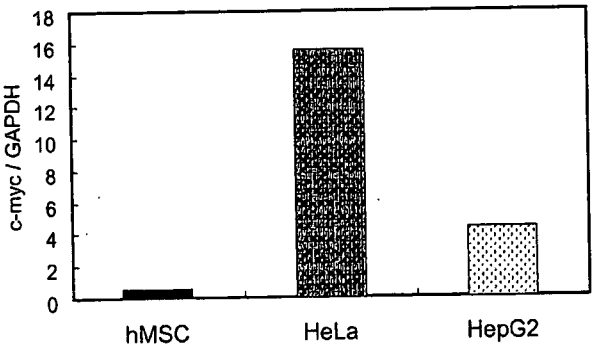

Fig. 2. The mRNA expression c-myc oncogene in hMSC, HeLa, and HepG2.

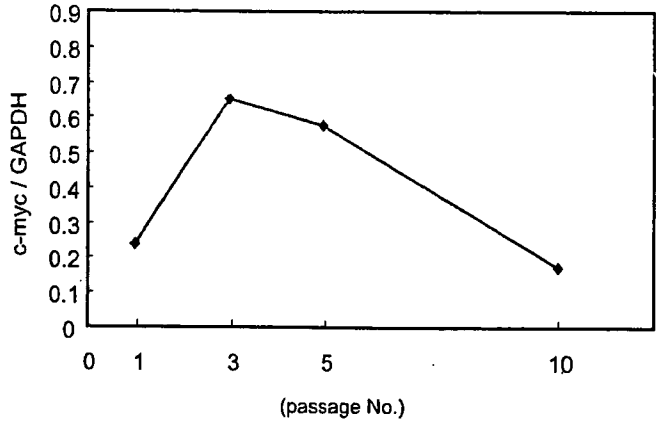

Fig. 3. Effect of the passage number on the mRNA expression c-myc oncogene in hMSC.

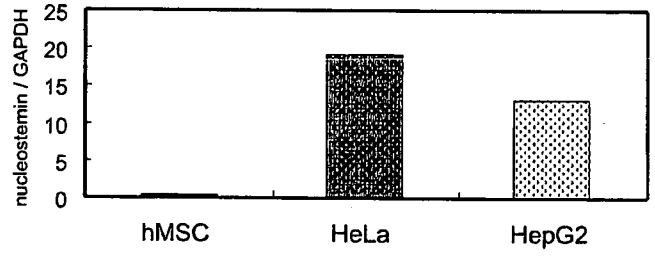

Fig. 4. The mRNA expression nucleostemin in hMSC, HeLa, and HepG2.

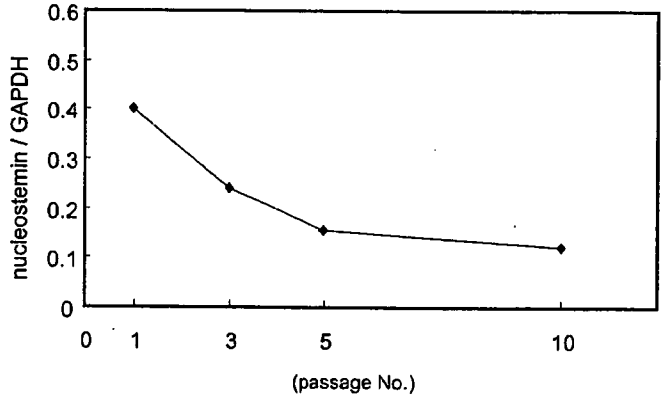

Fig. 5. Effect og the passage number on the mRNA expression of nucleostemin in hMSC.

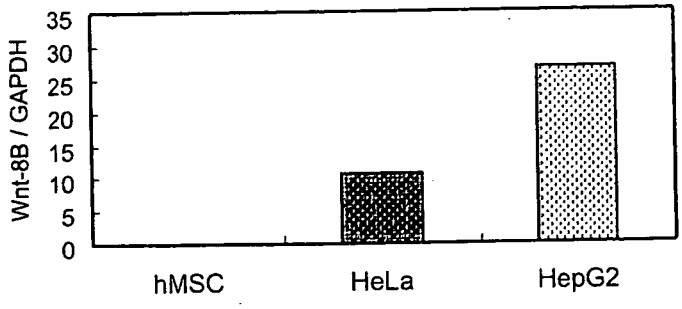

Fig. 6. The mRNA expression Wnt-8B in hMSC, HeLa, and HepG2.

4. DISCUSSION

In this study, effects of the passage number on the gene expression in hMSC were investigated. At first, c-myc oncogene and Wnt-8B concerned with cell proliferation and tumorigenesis were noticed by gene chip analysis (data not shown). Therefore, c-myc oncogene and Wnt-8B mRNA expressions in four kinds of passage numbers (#1, #3, #5, and #10) of hMSC were measured by quantitative real time RT-PCR. Furthermore, nucleostemin that concerned with proliferation of both stem cells and tumor cells (1) was also investigated. The proliferation speed of hMSC was lowered with the cell passage number (Fig. 1).

The mRNA expressions of c-myc oncogene, Wnt-8B, and nucleostemin in 1st, 3rd, 5th, and 10th passage of hMSC were investigated using quantitative real time RT-PCR. The mRNA levels of c-myc oncogene were decreased with the passage number from 3rd to 10th (Fig. 3). The mRNA expression of nucleostemin was decreased with the passage number (Fig. 5). In all three genes, their mRNA expressions of the stem cells (hMSC) were significantly lower than two kinds of tumor cells (HeLa and HepG2) (Fig. 2, 4, and 6). In hMSC, Wnt-8B was not expressed in any passage numbers. Although these results suggest that 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.

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EFFECT OF BIODEGRADABLE POLYMER POLY (L-LACTIC ACID) ON THE CELLULAR FUNCTION OF HUMAN ASTROCYTES

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Abstract: The objective of this study is to assay the efficiency and safety of poly (L-lactic acid) (PLLA) on human neural tissues. We used normal human astrocytes (NHA) to clarify effects of PLLA on their proliferation and differentiation. We cultured NHA with PLLA for one week, and determined NHA cell number and neural cell specific marker genes to assay their proliferation and development, respectively.

Cell proliferation was determined by tetrazolium salt (MTT) assay. The cell number of astrocytes cultured with 50 μg/ml PLLA was 70% of control. It has been suggested that a part of astrocytes had neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte. We compared gene expression of neural cell specific markers. Expression of Nestin, a specific gene for neural precursor cell was decreased in a dose-dependent manner, while expression of specific genes for neuron markers and astrocyte markers were not different from that of control.

PLLA suppressed astocyte proliferation in dose dependent manner. A neural precursor cell marker decreased when astrocytes were cultured with PLLA. These findings suggest that PLLA reduces proliferation and developmental potential of astrocytes.

Key words: Astrocyte, PLLA, proliferation, development

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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.

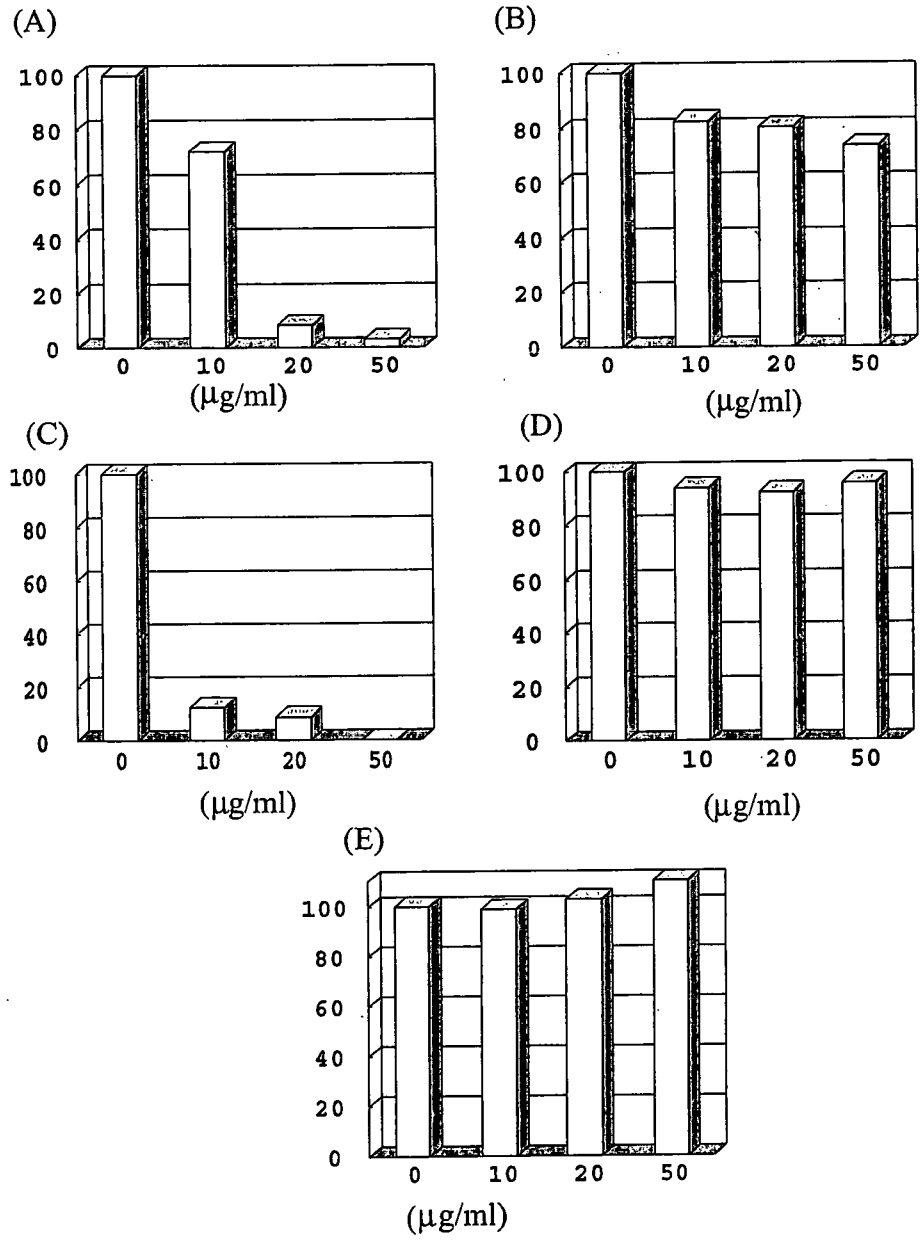

Fig. 1 Effect of PLLA on NHA proliferation (A) PLLA 3.000 (B) PLLA 5.000 (C) PLLA 11.000 (D) Lactic acid (E) Tin chloride

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

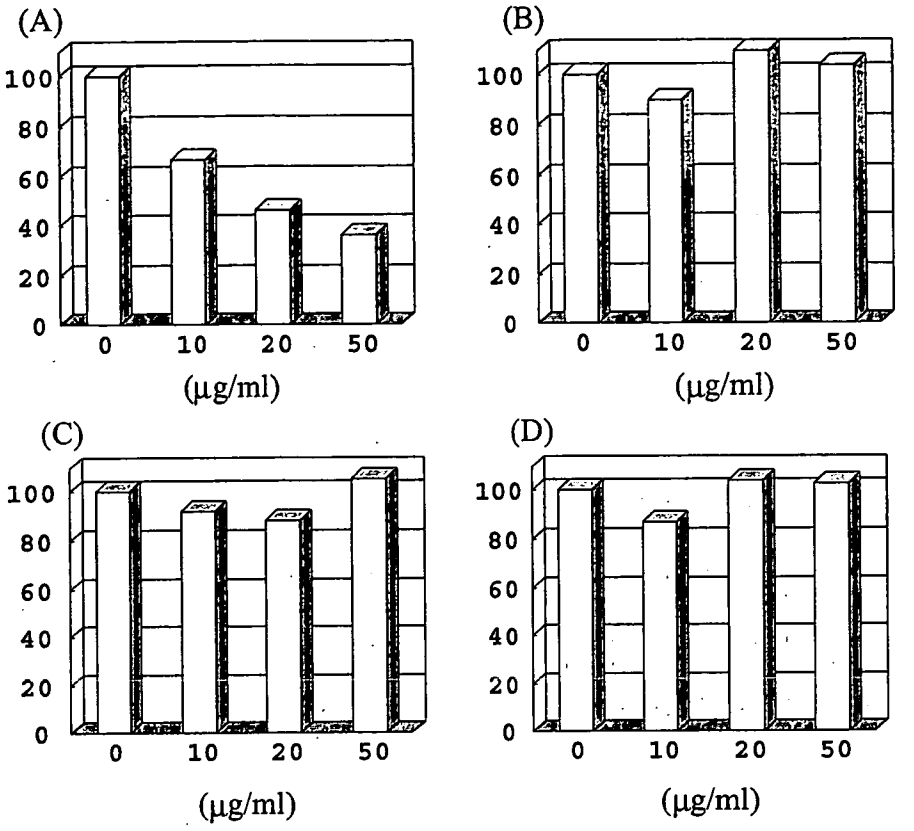

Fig. 2 Effect of PLLA on neural specific gene expression.
(A) Nestin (B) Nurr-1 (C) GFAP (D) Id-3

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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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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A polyelectrolyte complex (PEC) is a compound made from an electrically neutralized molecular complex of polyanions and polycations [13]. PEC can be prepared in various forms such as a film (2D) and a hydrogel, a microcapsule or a sponge (3D), which can be used as a scaffold in tissue regeneration studies. The effects of PEC films composed of polysaccharides on cell behavior have been studied, and we have already reported that PEC can stimulate differentiation of osteoblasts and periodontal ligament fibroblasts [14–16]. These studies suggest that PEC can be used as a biomaterial for repairing or regenerating tissues. In addition, because the PEC are composed of polysaccharides, PEC is expected not to elicit immune responses against it and to have better biocompatibility with the human body, although this is yet to be proved. Therefore, it is necessary to study the interactions between PEC and cells, especially human-derived, to clarify the usefulness of PEC as a biomaterial.

In this study, normal human osteoblasts (NHOst) were cultured on various PEC prepared on a tissue culture plate from chitosan as the polycation and modified chitins or hyaluronan as the polyanion. It should be generally agreed that estimating not only functional advantages but also safety and biocompatibility of biomaterials is important to develop them for clinical use, but the latter is not always studied. Therefore, we measured changes in gap junctional intercellular communication (GJIC) as well as the cell number and differentiation. GJIC is very important function for almost all cells to maintain their homeostasis [17]. During this decade, we have studied the effects of model biomaterials on the GJIC of cells cultured on them and suggested a possibility that changes in the GJIC can be used as an index of biocompatibility of biomaterials [18–21]. Therefore, we measured changes in GJIC of NHOst on PEC in order to estimate the biocompatibility of PEC from their effects on these cell functions.

2. Materials and methods

2.1. Chemicals

Fig. 1 shows the chemical structures of the polyanions and the polycation. Chitosan as the cationic polysaccharide and carboxymethylated chitin [CM-Chitin: degree of substitution (DS) = 1.0 (1.0 anionic site/saccharide ring)] were purchased from Katokichi Co., Ltd. (Kagawa, Japan). Sulfated chitin (S-Chitin: DS = 1.5), phosphated chitin (P-Chitin: DS = 1.6), hyaluronan (HA), and sulfated hyaluronan (SHA: DS = 1.05) were prepared as previously reported [14–16,22].

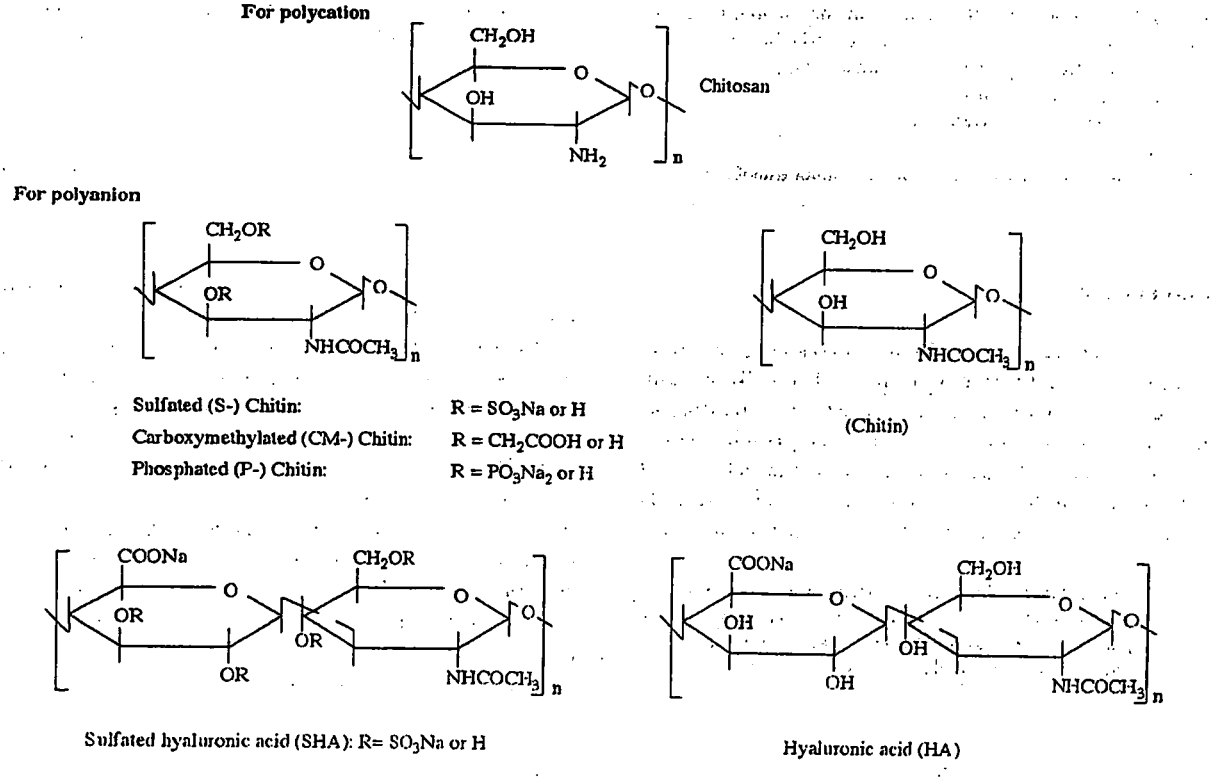

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 20 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 4 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test, Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

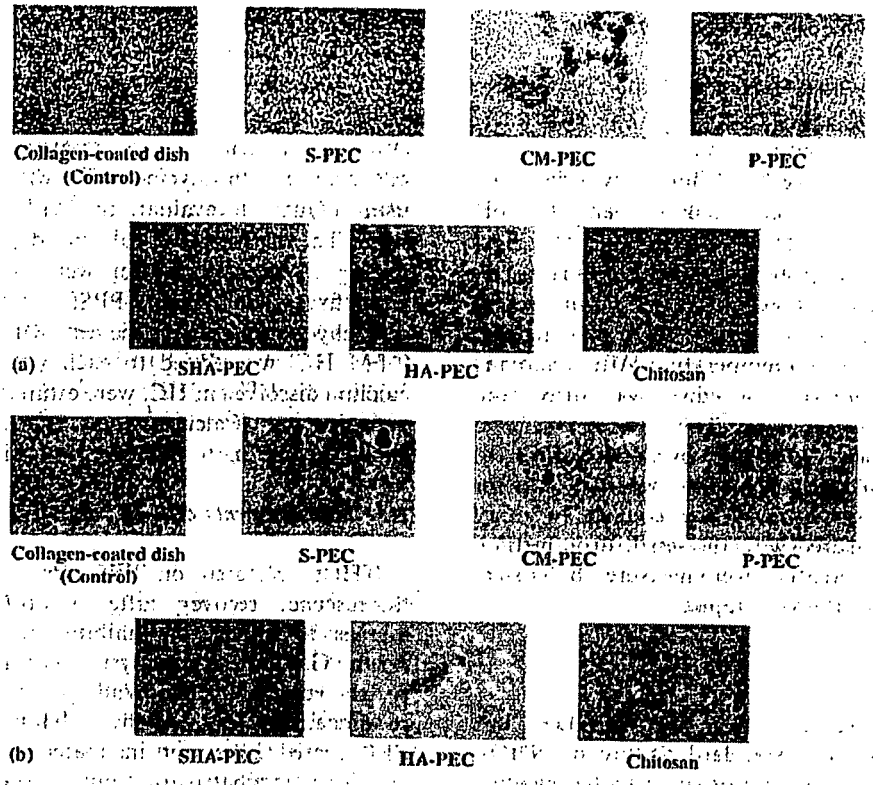

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

After 2-day incubation, the NHOst on PEC composed of chitosan and either sulfated chitin (S-PEC) or sulfated hyaluronan (SHA-PEC) showed morphologies similar to those on a normal culture plate. When cells were cultured on PEC of chitosan and phosphated chitin (P-PEC), some of them formed small aggregates, while the rest showed morphologies similar to those on S-PEC and SHA-PEC. On the other hand, NHOst cultured on PEC from chitosan and either carboxymethyl chitin (CM-PEC) or hyaluronan (HA-PEC) did not adhere well and showed aggregation. Similar morphologies of the cells on the PEC were observed after 1 day of incubation (data not shown). Even after 1 week of incubation, the morphologies and attachment of the cells on the PEC films did not change (Fig. 2). Only cells grown on cationic polysaccharide chitosan-coated culture dishes preserved morphology of very similar to NHOst grown on collagen-coated cultured dishes, indicating that these morphological differences are ascribable to differences in the anionic polysaccharides of which the PEC is composed.

It has been reported that cell attachment, morphology, and response are influenced by physico-chemical properties of the material surface [23,26]. To clarify what properties of PEC control the attachment and morphology of the cell, the contact angle and zeta

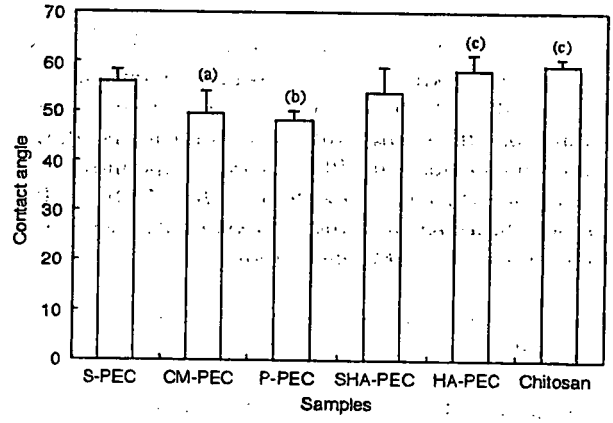

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

potential of PEC films were estimated. Although their compositions are different, large differences in their contact angles were not observed (Fig. 3). On the other hand, a measurement of zeta potentials of the PEC showed interesting results (Table 1). The measurement revealed that S-PEC and SHA-PEC have negative zeta potentials, whereas PEC films made of polysaccharides