

2. Materials and methods

2.1. Preparation of recombinant P2X₂ receptor protein

N-terminal hexahistidine-tagged recombinant rat P2X₂ receptor was expressed using baculovirus-Sf9 system, which has been used for the expression of membrane receptor proteins (e.g., Boundy et al., 1993; Ng et al., 1993). cDNA encoding rat P2X₂ receptor (Brake et al., 1994) was subcloned into pFast BAC HTc vector (BD Bioscience Clontech, Palo Alto, CA, USA). The recombinant virus was transfected to insect-derived clonal Sf9 cells. After culturing at a volume of 500 ml at room temperature, the culture medium was centrifuged at 130 ×g for 5 min, and the precipitated cells were washed with Ca²⁺, Mg²⁺-free phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄; PBS(-)) twice. The cells were then suspended in a Tris-HCl (pH 7.4) lysis buffer containing Triton X-100, and homogenized. NaCl was added such that its final concentration became 100 mM. This solution was centrifuged at 30000 ×g for 20 min, and the supernatant was then centrifuged at 380000 ×g for 10 min. Polyacrylamide gel electrophoresis followed by immunoblotting analysis with anti-hexahistidine antibody showed that hexahistidine-tagged proteins of an expected size (56 kD) were found in this supernatant. Further purification was made using Chelating Sepharose FF columns. Ni²⁺-bound columns were equilibrated with a buffer containing 20 mM Tris-HCl and 0.5 M NaCl (pH 8.0), and samples were applied. The bound receptor proteins were eluted by stepwise increase of imidazole (10, 20, 50, 100, 200 and 500 mM). The concentrations of the receptor protein in the eluted solutions were estimated by measuring absorbance at 595 nm. The most purified P2X₂ receptor protein (>90% of total protein) was found in the fraction eluted by 10 mM imidazole, and this fraction was served for atomic force microscopy imaging. The purified P2X₂ receptor exhibited the ability to bind ATP when photoaffinity labeling with [α -³²P]ATP was performed according to Kim et al. (1997). In this experiment, the binding of [α -³²P]ATP was markedly reduced by 100 μ M nonradiolabeled ATP.

2.2. Atomic force microscopy imaging

The protein solution (about 1.5 μ M) was diluted to appropriate concentrations (0.1 to 10 nM) with water, and the diluted solution was placed on freshly cleaved mica. After 30 min, unbound proteins were washed away with water, and served for atomic force microscopy imaging. When ATP (disodium salt; Sigma, St. Louis, MO, USA) was added to water, 1 mM solution was neutralized to pH 7.4 with 2 N NaOH (final Na⁺ concentration was about 16 mM). In part of the experiments, the protein solution was diluted with a Tris buffer of the following composition (in millimolar): Tris 50, KCl 150, MgCl₂ 10, dithiothreitol 1 (pH 7.0). This buffer composition was similar to that utilized for atomic force microscopy imaging of *Escherichia coli* GroES (Cheung et al., 2000). Imaging was made in an aqueous tapping mode using MFP-3D (Asylum Research, Santa Barbara, CA, USA) equipped with OMCL-TR800PSA (Olympus, Tokyo, Japan) as a probe.

3. Results

Fig. 1A shows atomic force microscopy images of purified P2X₂ receptor proteins in water. A larger part of the proteins were found as dispersed particles. The height of single P2X₂ receptor

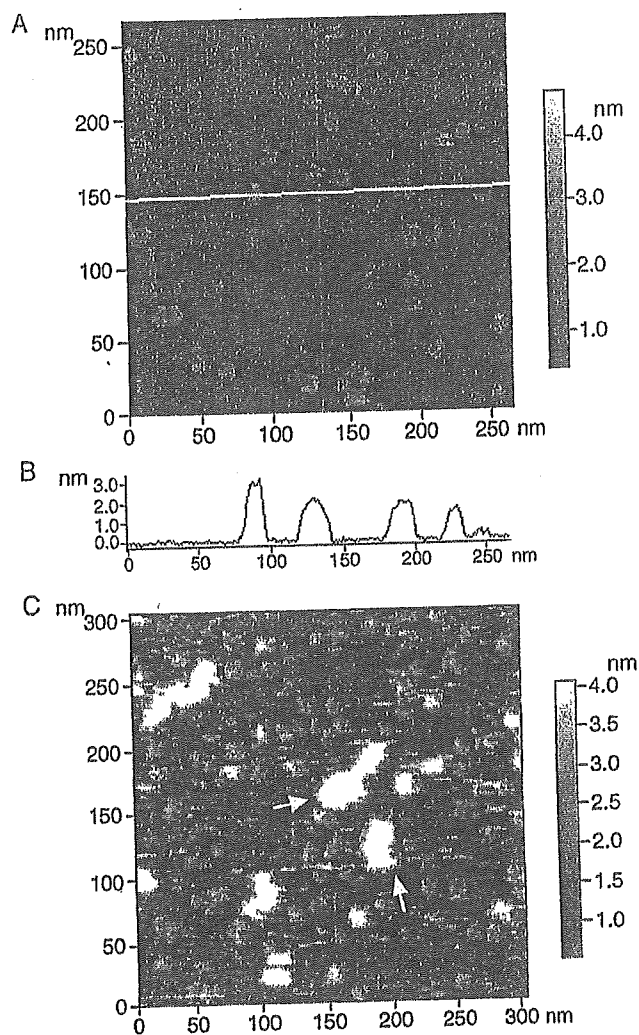


Fig. 1. (A) An atomic force microscopy image of P2X₂ receptor proteins in water. Isolated single receptor proteins and their small assemblies are seen. (B) A section of the image shown in (A). The section was made along with the line. The height of receptor proteins is about 3 nm or less. (C) An image of P2X₂ receptor proteins in the presence of 1 mM ATP. In addition to single receptor proteins, clots of the proteins (indicated by arrows) were also seen.

proteins was about 3 nm (Fig. 1B). In the presence of 1 mM ATP, larger particles, presumably clots of several receptor proteins, were observed in addition to dispersed particles (Fig. 1C). A flatly and densely assembled image was obtained when the proteins were dissolved in a Tris buffer containing 1 mM ATP (Fig. 2A). Densely packed assembly is advantageous for atomic force microscopy imaging because resolution is improved due to smaller movement of probes along Z-axis (Müller and Engel, 2002). When the protein assembly shown in Fig. 2A was imaged at higher magnification, a circular structure with a pore was observed (Fig. 2B). The diameter of the circular structure was about 10 nm, and that of the pore was several nanometers. Without ATP, the protein was not densely assembled and did not exhibit uniform direction (not shown).

4. Discussion

For atomic force microscopy imaging of membrane proteins, densely expressed proteins in particular cells have

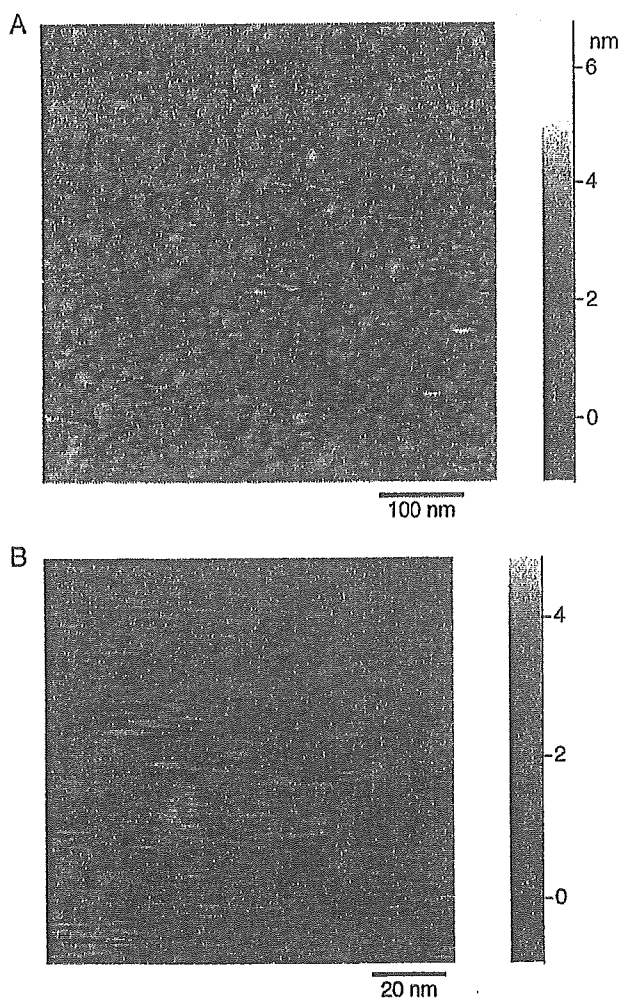


Fig. 2. (A) An image of P2X₂ receptor proteins in a Tris buffer containing 1 mM ATP. The proteins were flatly and densely assembled. (B) An expanded image. The upper surfaces of individual proteins exhibited a circular structure having a pore in its center.

been served in the presence of lipid bilayers (Müller and Engel, 2002; Müller et al., 2002). This two-dimensional (2D) protein crystal can provide high resolution of images, especially when combined with image processing including averaging. However, preparation of 2D crystals requires skilled techniques or special equipment. The present study has shown that recombinant P2X₂ can be imaged by atomic force microscopy without special techniques, but by simply adding agonist molecule, ATP. The role of ATP in promoting the densely packed assembly is unclear at present. It is speculated that receptor protein molecules without ATP freely move and exhibit various conformations, whereas ATP-bound receptor molecules exhibit only one or a restricted number of conformations in aqueous phase. The pore identified in the center of the protein may be the ion channel involved in P2X₂ receptor. A similar pore has been observed in connexin that also forms ion channels (Müller et al., 2002). It is unclear that the pore corresponds to the inner mouth or the outer mouth of the channel. Nevertheless, it is interesting that a number of proteins appear to exhibit similar

surface structure (Fig. 2B). P2X₂ receptor possesses a large extracellular domain, and, thus, it is possible that this domain is orientated upward to increase contact with the aqueous phase. Atomic force microscopy imaging of isolated membrane proteins may be less advantageous to elucidate biological functions compared to those embedded in lipid bilayer. However, isolated proteins are more readily observed than membrane preparations, and the imaging of these proteins may provide insights into the intrinsic properties of proteins and useful information to clarify the interactions between proteins and the membrane.

Barrera et al. (2005) observed dried P2X₂ receptor protein as a simple particle. We have revealed the outer structure of the protein, suggesting that resolution was better in the present study. However, our observation has not resolved trimeric assembly of P2X₂ receptor protein, which has been shown using antibodies specific for the protein (Barrera et al., 2005). Trimeric assembly of P2X₂ receptor has been also demonstrated by electrophysiological and biochemical studies, and two transmembrane regions of each subunit are believed to contribute to the forming of channel pore (North, 2002; Vial et al., 2004). If P2X₂ receptor forms a six-barrel channel like connexin, this may account for a similar pore size (about several nanometers). Further improvement will be necessary to identify individual subunit proteins that form P2X₂ receptor and clarify more detailed structure by atomic force microscopy.

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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 $\mu\text{g}/\text{well}$) were subjected to the assays.

Cellular Assays. Normal human osteoblasts (NHOst) were purchased from BioWhittaker 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).

All assays were carried out using the medium supplemented with 10mM β -glycerophosphate. NHOst (2×10^4 cells/well/500 μ l 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 μ g/500 μ l 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'CATGAGAGCCCTCACAA3' 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 \pm 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 μ m and 5 μ m 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 0.1 μ m 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 μ m PS microspheres suggests that not only the area they occupied but also their size may cause the unique inhibitory activity of the 5 μ m 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

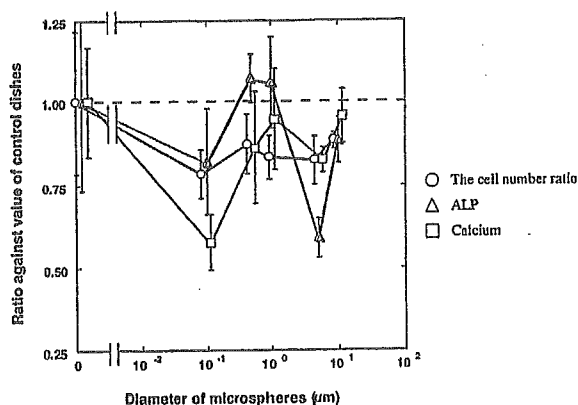


Figure 1. Effects of diameter of pre-coated PS on various functions of NHOst. Data are shown as the means \pm SD

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 phagocytose PS microspheres as well as macrophages, and in particular, may phagocytose 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 re-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 $\mu\text{g}/\text{well}$)

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

Data are shown as the mean value \pm SEM (n = 4 to 22)

* p < 0.01, against control group

(a) p < 0.05, against NHOst co-cultured with polyethylene and alumina microspheres

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.

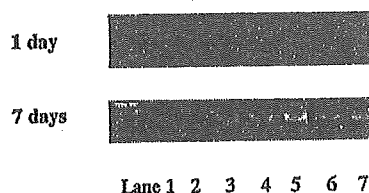


Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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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 (NH₂Ost). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NH₂Ost on it although the NH₂Ost on it showed an enhancement in their differentiation level. On the other hand, NH₂Ost on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NH₂Ost 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, NH₂Ost 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 NH₂Ost to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NH₂Ost 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.

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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 (NH₂Ost) 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 NH₂Ost 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].

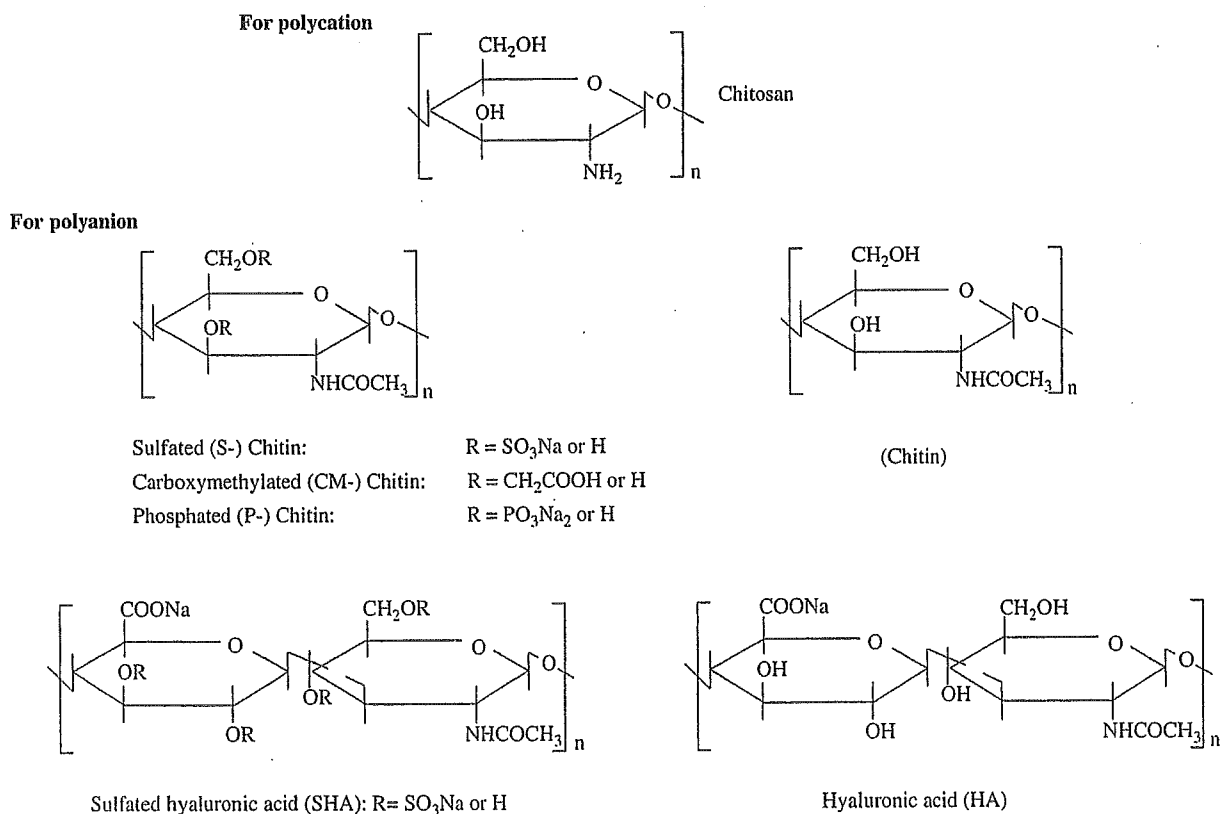


Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

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

NH0st were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NH0st 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% CO₂–95% air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NH0st 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 NH0st cultured on PEC films

The proliferation of NH0st 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 NH0st 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 MgCl₂, 0.1 mM ZnCl₂ 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

NH0st 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, NH0st 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 Ca²⁺ and Mg²⁺ (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 NH0st 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 NH0st 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 NH0st adhering to PEC films.

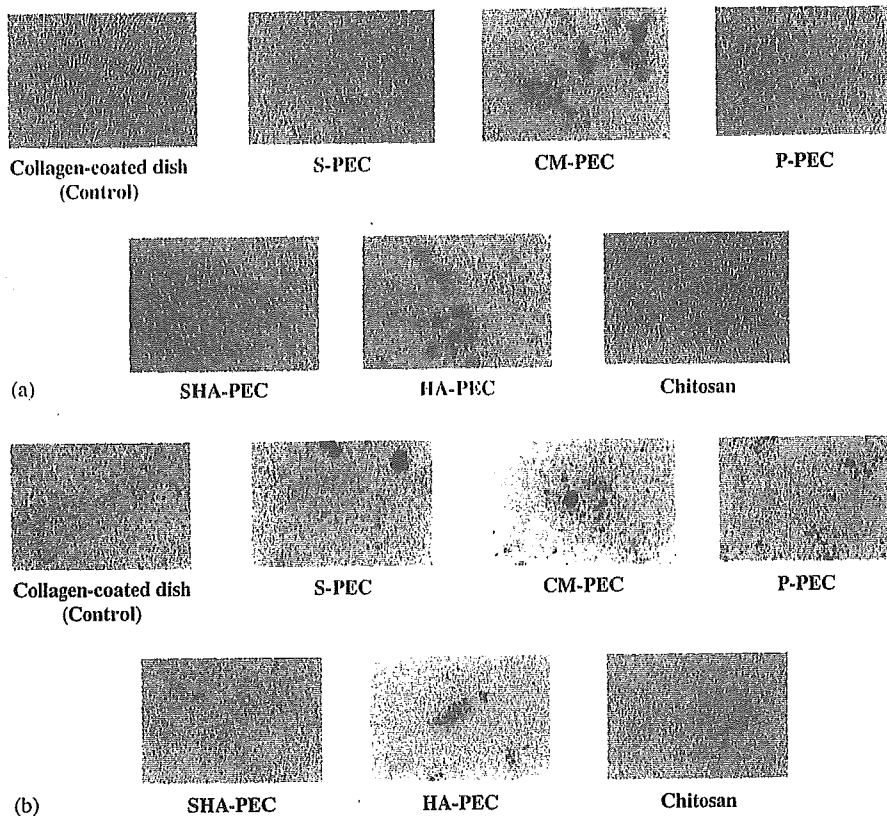


Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: $\times 100$).

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

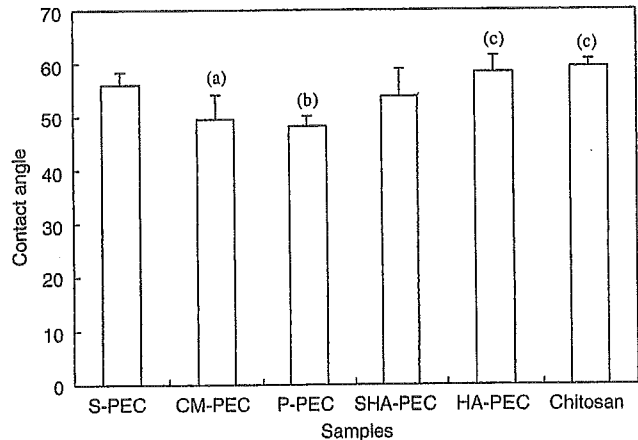


Fig. 3. Contact angles of PEC films studied: (a) $p < 0.05$ against S-PEC, (b) $p < 0.01$ against S-PEC, (c) $p < 0.01$ against both CM-PEC and P-PEC.

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

Table 1
Zeta potentials of various PEC prepared on a culture dish

| | Culture | S-PEC | CM-PEC | P-PEC | SHA-PEC | HA-PEC |
|---------------------|---------|-------|--------|-------|---------|--------|
| Zeta potential (mV) | -58.7 | -28.0 | 34.5 | 24.9 | -5.7 | 29.5 |

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

| Samples | The cell number (percent against control) | ALP activity The cell number (ratio) | Ca amount The cell number ($\mu\text{g}/\text{ratio}$) |
|----------------------|-------------------------------------------|-----------------------------------------|-------------------------------------------------------------|
| Collagen-coated dish | 100.0 \pm 17.0 | 1.00 \pm 0.15 | 3.4 \pm 0.5 |
| S-PEC | 82.2 \pm 6.1 | 0.98 \pm 0.11 | 10.7 \pm 3.6 |
| CM-PEC | 6.0 \pm 2.6* | 0.05 \pm 0.08* | 27.4 \pm 3.0* |
| P-PEC | 130.4 \pm 6.3 | 0.02 \pm 0.01* | 2.5 \pm 0.8 |
| SHA-PEC | 71.4 \pm 22.1 | 1.35 \pm 0.48 | 2.1 \pm 1.0 |
| HA-PEC | 8.1 \pm 3.0* | 0.52 \pm 0.31 | 38.3 \pm 12.3* |
| Chitosan | 79.5 \pm 25.0 | 0.93 \pm 0.13 | 2.7 \pm 2.0 |

* $p < 0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from -30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were

cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in simulated body fluid [27]. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP

activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC does not affect NHOst functions. Actually, there were no statistical differences in the amounts of calcium deposited between NHOst on the PEC and the collagen-coated dish although NHOst on S-PEC showed higher average calcium deposition. Thus, it is suggested that the PEC films made from sulfated polysaccharides are comparable substrates to a collagen-coated dish for cell culture. When compared to a normal culture dish, it has been reported that S-PEC can induce aggregation of cultured human fibroblasts and enhance their DNA synthesis in an earlier stage of cell culture by activation of the ERK pathway [28]. Since we used a collagen-coated dish as a control in this study, it is expected that the pathway of NHOst on the dish may be already activated through integrin molecules on the NHOst membrane. Therefore, the results in this study suggest the PEC from sulfated polysaccharides have a potential to proliferate and differentiate NHOst very similar to that of collagen.

To assess the effects of PEC films on cell function, gap junctional intercellular communication (GJIC), which is an important function of cells for maintenance of homeostasis [17], of NHOst on the films were measured. As shown in Fig. 4, GJIC of NHOst on PEC films did not show statistically significant differences compared to those grown on a collagen-coated dish. Although the GJIC of NHOst on CM-PEC showed a decrease after 1 day of incubation, it had recovered after 1 week. This result suggests that most PEC films have the potential to maintain homeostasis of attached cells although they showed different influences on the number and the

differentiation of NHOst. On the other hand, NHOst on chitosan, which was used as the polycation for all PEC, showed suppression of GJIC after 1 week. This suggests that chitosan disturbs homeostasis maintenance of NHOst, but improve its biocompatibility by forming PEC films with other anionic polysaccharides. Therefore, PEC might be used as a biocompatible material for medical devices and tissue engineering scaffolds.

4. Conclusion

PEC films composed of various polysaccharides were prepared, and their effects on NHOst functions were evaluated. Attachment, morphology, growth and differentiation of NHOst were influenced by the composition of the PEC on which they were grown. NHOst attachment decreased and their aggregates were observed on PEC prepared from polysaccharides containing a carboxyl group (CM- and HA-PEC). ALP activity of NHOst was suppressed on these PEC films although calcium deposition was observed more frequently than on other PEC films. In addition, these PEC films strongly suppressed proliferation of NHOst. PEC prepared from phosphated chitin and chitosan (P-PEC) showed low ALP activity and calcium deposition, although the number of NHOst was highest after 1-week incubation. These indicate unsuitability of these three PEC for usage in tissue engineering. On the other hand, NHOst adhered to and proliferated well on PEC films when sulfated polysaccharides were used as the polyanion (S- and SHA-PEC). Moreover, these PEC films showed almost the same suitability as the collagen-coated dish in all cell functions studied, indicating that these PEC films, especially S-PEC can be used as a scaffold for bone regeneration. Further studies, especially in vivo studies, are needed to clarify the usefulness of PEC films for tissue engineering.

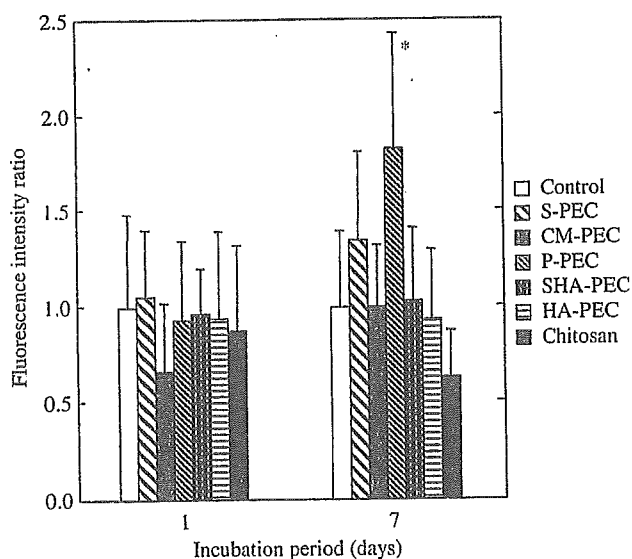


Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (* $p < 0.01$ against control).

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Hydroxy apatite microspheres enhance gap junctional intercellular communication of human osteoblasts composed of connexin 43 and 45

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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 from the joint. To assess the effect of wear debris on osteoblasts, effects of model wear debris on gap junctional intercellular communication (GJIC) of normal human osteoblasts were estimated. The GJIC activity of the osteoblasts after a 1-day incubation with the microspheres was similar to that of normal osteoblasts. However, hydroxy apatite particles, which have been reported to enhance the differentiation of osteoblasts in contact with them, enhanced the GJIC function of the osteoblasts. From RT-PCR studies, not only connexin 43 but also connexin 45 is suggested to play a role in the GJIC of the osteoblasts in an early stage of

coculture with the microspheres, although it is still unclear how these connexins work and are regulated in the GJIC and differentiation. However, this study suggests that there is a relationship between the early levels of GJIC and the differentiation of the cells. Therefore, estimating the effect of biomaterials, even in the microsphere form, on the GJIC of model cells, with which the biomaterials may be in contact *in vivo*, can provide important information about their biocompatibility. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 74A: 181–186, 2005

Key words: gap junctional intercellular communication; human osteoblasts; microspheres; hydroxy apatite; connexin

INTRODUCTION

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, sometimes resulting in undesirable side effects. One well-known example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. It has already been 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.^{1–14} Macrophages stimulated by wear debris *in vitro* release significant amounts of inflammatory mediators such as interleukin-1, interleukin-6, prostaglandin E2, collagenase, and tumor necrosis factor.^{6–14} In addition, the biological effects of wear debris may depend on the type of material used as well

as the shape, size, and amount of the debris.^{4–11} Therefore, it is important to estimate the biocompatibility of biomaterials with not only their original shape but also possible transformed shapes after their usage.

During the last decade, we have been researching the inhibitory potential of many kinds of biomaterials on gap junctional intercellular communication (GJIC) as an index for their biocompatibility.^{15–18} GJIC is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules, which results in regulating cell growth, development, and differentiation of cells.^{19,20} Therefore, it is reasonable that disruption of this function is the cause of many kinds of diseases. In a previous report,¹⁸ we examined the inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer *in vivo*, on the GJIC of rodent-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in the body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joints by estimating their effect on GJIC function.

No benefit of any kind will be received either directly or indirectly by the authors

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However, it must be noted that the effects of the microspheres may be different when the effects on the GJIC of human-derived cells are estimated. 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.^{21,22} Therefore, the question is raised whether wear debris has an inhibitory effect on the GJIC and the GJIC inhibition has a relation with the aseptic loosening of artificial joints. Because we have already observed some precoated polymer microspheres around 5 μm in diameter showed the potential to inhibit GJIC of fibroblasts contacting with them,²³ we estimated effects of various microspheres around 5 μm in diameter on GJIC function using normal human osteoblasts to discuss the relationship between the GJIC and the differentiation of osteoblasts. In this study, we employed fluorescence recovery after photobleaching (FRAP) analysis for estimating the GJIC function,¹⁷ and assessed the potential effect of many kinds of microspheres on the GJIC.

MATERIALS AND METHODS

Microspheres

Monodispersed polystyrene (PS) microspheres (5 μm in diameter) were purchased from Japan Synthetic Rubber Co., Ltd. (Tokyo, Japan). Low-density polyethylene (PE) microspheres were generously supplied 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 hydroxy apatite microspheres (HA, 7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. A Multisizer II (Coulter Electronics Inc., Hialeah, FL) was used to determine the average diameter of PE and alumina microspheres: 6.4 and 5.1 μm , respectively. Microspheres were sterilized by dispersing them in a 70% ethanol solution, followed by centrifugation in sterile conditions to remove the ethanol solution. The microspheres were dispersed in sterile methanol for cell differentiation tests at specified concentrations. The suspension of microspheres in methanol was added to 35-mm type I collagen-coated cell culture dishes (Asahi techno glass, Chiba, Japan), and the plates dried overnight at room temperature. The obtained microsphere-coated dishes (100 μg /dish) were subjected to the assays.

Cell culture

Normal human osteoblasts (NHOst) were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco) 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 (1×10^5 cells/dish/2.5 mL medium) were cultured on microsphere-coated dishes for estimating the effect of the microspheres interacted from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst cells were cultured with microsphere-containing medium (100 μg /2.5 mL medium) after they had adhered to the collagen-coated dishes. The test cells were cultured while changing the medium three times when the measurement of GJIC was performed after a 7-day incubation.

Measurement of GJIC activities

NHOst cultured with microspheres were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these microspheres toward the GJIC. FRAP analysis was carried out according to an original procedure by Wade et al.,²⁴ with some modifications.¹⁷ Briefly, NHOst were plated on microsphere-coated dishes and incubated for 1 or 7 days. After a wash with phosphate buffer saline (PBS) containing MgCl_2 and CaCl_2 [PBS(+)], the cells were incubated for 5 min at room temperature in PBS(+) containing 5,6-carboxyfluorescein diacetate (7 μg /mL, excitation 488 nm and emission 515 nm). After the washing off of excess extracellular dye with PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. In the control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure as the tested cells. Cells in contact with test microspheres and at least two other cells were subjected to FRAP analysis under an Ultima-Z confocal microscope (Meridian Instrument, Okemos, MI) with a 10 \times objective lens at room temperature. The cells were photobleached with a 488-nm beam and the recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total period of 4 min. The data obtained from more than seven independent cells were expressed as the average of fluorescence recovery rate in comparison to the rate obtained from NHOst cultured without microspheres.

Effect of microspheres on calcium deposition by NHOst

The amount of calcium deposited during a 7-day incubation of the cells were evaluated as follows: NHOst were cocultured with either precoated or added microspheres in 24-well collagen-coated culture plates (Asahi techno glass, Chiba, Japan) for 1 week (2×10^4 cells/20 μg microspheres/well/500 μL medium). After the cells were fixed in formaldehyde, 0.5 mL of 0.1 M HCl was added to each well after washing the cells with PBS. The amounts of calcium dissolved in HCl were estimated using a Calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to the manufacturer's direction.

RT-PCR for estimating expression of connexins

According to the method reported by Ichikawa et al.,²⁵ RT-PCR was performed to detect the expression of connexin mRNA in NHOst. After culturing NHOst with microspheres for a scheduled time, total RNA was extracted from the NHOst using TRIZOL[®] reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. After dissolving the RNA in diethylpyrocarbonate-treated water, the total RNA concentration was measured spectrophotometrically using Genequant (Amersham Biosciences Corp., Piscataway, NJ). RNA samples were adjusted to a minimum concentration among collected samples in each experiment and reversibly transcribed to cDNA using Superscript[™] II (Invitrogen Corp.). For PCR amplification of human connexin 45, Takara Ex-Taq[™] (Takara Shuzo Co., Ltd., Shiga, Japan) was used with Ex-Taq[™] buffer consisting of 20 pmol each of two human connexin-45 specific primers (forward 5'GTGGCAACTCCCTCTGTGAT3' and reverse 5'GGATCCTCAAGTTCCTCCT3'). For PCR amplification of human connexin 26, 32, and 43, Takara LA-Taq[™] (Takara Shuzo Co., Ltd.) was used with Ex-Taq[™] buffer consisting of 6 pmol each of the human connexin-specific primers (for connexin 26, forward 5'ATGGATTGGGGCACGC3' and reverse 5'TTAAACTGGCTTTTTTGACTTCCC3'. For connexin 32, forward 5'ATGAACTGGACAGGTTTGTACACCTTGCTC3' and reverse 5'TCAGCAGGCCGAGCAGCGG3'. For connexin 43, forward 5'ATGGGTGACTGGAGCGCCTTAGGC3' and reverse 5'CTAGATCTCCAGGTCATCAGGCCG3'). The PCR profile for connexin 45 involved pretreatment at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 90 s. The PCR profile for connexin 26, 32, and 43 (35 times) was as follows: pretreatment at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 120 s. Reaction products were analyzed by electrophoresis in 1.5% (w/v) agarose gel, followed by staining of

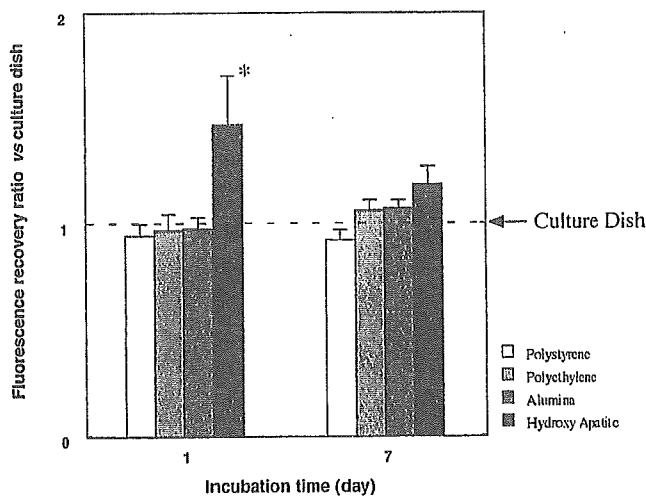


Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (**p* < 0.01 against culture dish).

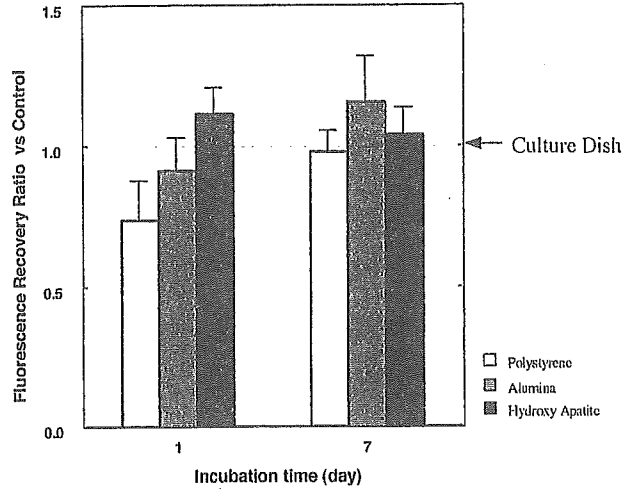


Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

the products by SYBR[®] Green I (Takara Shuzo Co., Ltd.) and detection of a 566-bp (connexin 45), 671-bp (connexin 26), 852-bp (connexin 32), and 1149-bp (connexin 43) band, respectively. For the standardization of connexin cDNA, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample was performed using GAPDH-specific primers (forward 5'CCCATCACCATCTTCCAGGAGCGAGA3' and reverse 5'TAAGTAGGACAA-CAAGGAGGTCGTGACGACGC3'; product size 578-bp). All reactions included negative controls without cDNA.

Statistical analysis

All data were expressed as the mean value ± the standard error of the means of the obtained data and treated statistically with Student's *t* test.

RESULTS

Figure 1 shows effects of various microspheres on GJIC of NHOst in contact with the microspheres for 1 and 7 days. The microspheres were precoated on 35-mm culture dishes before cell seeding. When the NHOst were cultured with precoated PS, PE, and alumina microspheres, their GJIC level was similar to that in NHOst cultured on a normal culture dish. On the other hand, the GJIC level was 1.5 times that of NHOst on the normal dish when they were cultured with precoated hydroxy apatite microspheres. After 7 days, the GJIC of NHOst in contact with microspheres became similar to that of normal NHOst, irrespective of the type of microsphere. The change in GJIC of NHOst in contact with added microspheres is shown in Figure 2. As seen in Figure 1, hy-

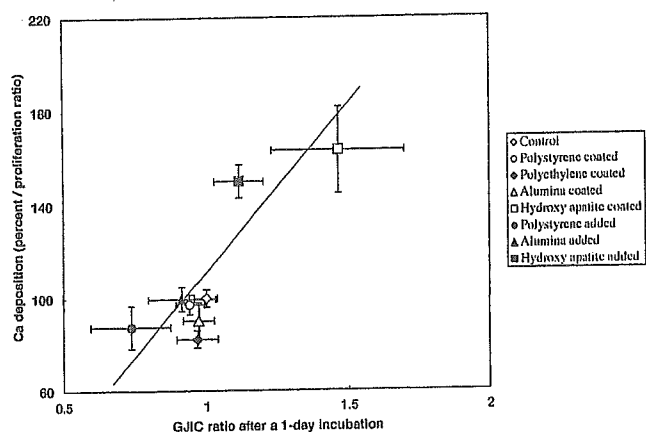


Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres ($r^2 = 0.74$).

droxy apatite microspheres enhanced their GJIC after a 1-day culture compared to cells on a normal plate. The degree of enhancement of GJIC is, however, smaller than that seen in NHOst in Figure 1, and no significant difference was observed between NHOst in contact with the hydroxy apatite microspheres and those cultured without microspheres. In addition, Figure 2 indicates that addition of PS microspheres into a culture of NHOst inhibited GJIC.

To consider the effects of tested microspheres on not only GJIC but also the differentiation of NHOst, changes in the amount of calcium deposited after a 1-week coculture of NHOst with various microspheres were estimated. From Figure 3, it is suggested that there is the possible relation between the GJIC of NHOst cocultured with microspheres for 1 day and

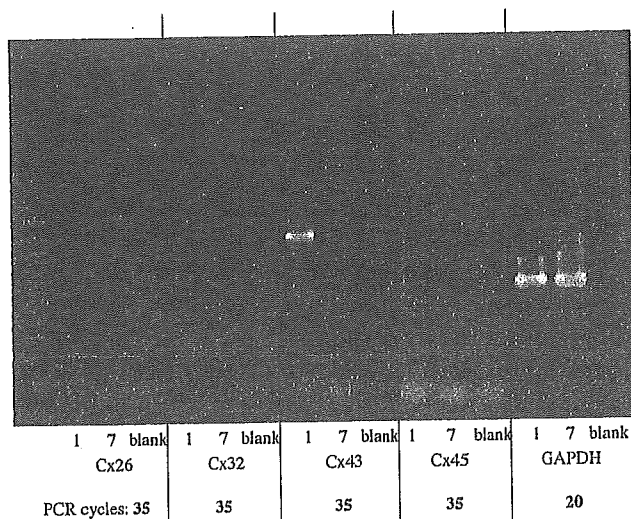


Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2×10^5 . RT-PCR cycles of each lane are expressed at the bottom of the figure.

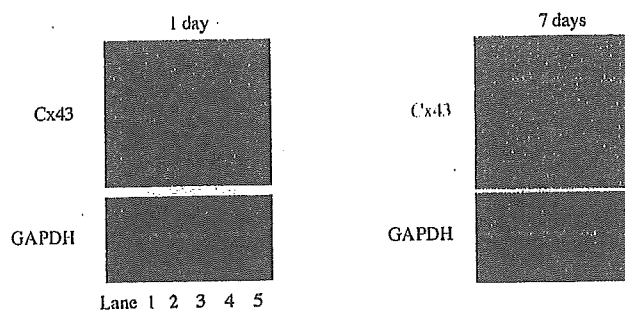


Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

the amount of calcium deposited after a 1-week coculture with the same microspheres.

To clarify which connexins exist in NHOst, we performed RT-PCR to detect mRNA of connexin 26, 32, 43, and 45 in NHOst cultured on a normal culture dish. Figure 4 shows the result of RT-PCR to amplify the mRNA from whole RNA collected from NHOst cultured for 1 and 7 days. As shown in the figure, only connexin 43 and 45 were detected in NHOst. When cells were cultured for 7 days, connexin 43 was detected at a lower level than that detected after the 1-day culture, while connexin 45 was not detected.

Figures 5 and 6 show the results of RT-PCR to amplify mRNA of connexin 43 and 45 in NHOst cultured with various precoated microspheres. The NHOst cultured with microspheres did not express mRNA of connexin 43, except those with PE microspheres. After 7 days, the expression was suppressed in the normal NHOst while the expression was observed in NHOst cultured with microspheres, irrespective of kind of the microsphere. On the other hand, mRNA expression of connexin 45 was suppressed after a 1-day culture of NHOst only with alumina microspheres, followed by a decrease in expression of the mRNA after their 7-day culture.

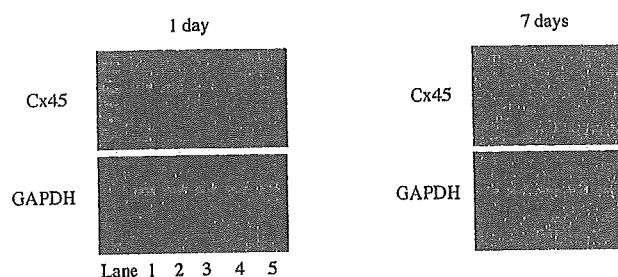


Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

DISCUSSION

As shown in Figure 3, normal human osteoblasts (NHOst) in contact with microspheres showed different levels of calcium deposition only after a 1-week culture, suggesting composition of the microspheres affects NHOst differentiation level. The differentiation was suppressed by the contact with PS, PE, and alumina microspheres, while HA microspheres showed the potential to enhance the differentiation. It has been reported that GJIC plays an important role in not only the homeostasis of cells but also their differentiation.¹⁹⁻²² In addition, GJIC is affected by the microsphere's composition, as has been reported using a fibroblast cell line.¹⁸ Therefore, the results shown in Figures 1 and 2 suggest that the enhanced differentiation of NHOst relates to GJIC enhancement on a 1-day culture in contact with HA microspheres, especially the precoated microspheres. In addition, on coculture with other microspheres, GJIC was slightly suppressed at 1 day, although no significant difference compared to control NHOst was observed. We have already studied effects of the microspheres on NHOst differentiation, and enhancement of calcium deposition by coculture with the hydroxy apatite microspheres was observed. Figure 3 suggests a relationship between the calcium deposition and GJIC on day 1. This also indicates that GJIC of the NHOst, in contact with materials in the microsphere form, in the early stage may be one factor affecting their differentiation.

It has been reported that GJIC of cells derived from human osteoblasts is mainly composed of connexin 43 and 45.^{22,26,27} In this study, it is also indicated that GJIC of NHOst is composed of connexin 43 and 45 (Fig. 4). Therefore, it is possible that changes in the level of their GJIC is ascribed to the change in mRNA expression level of connexin 43 and 45 and their expression ratio. From Figures 5 and 6, mRNA of connexin 43 was expressed only in normal NHOst and those cultured with PE microspheres, while it was slightly expressed in NHOst cocultured with HA. On the other hand, mRNA of connexin 45 was expressed in NHOst in all conditions, except those cocultured with alumina microspheres. Because HA was observed to enhance GJIC of NHOst, this suggests that connexin 45 may play a role in GJIC at an early stage. This also suggests that a higher level of connexin 45 than that of connexin 43 may be important in the enhancement of GJIC. However, although the mRNA expression of neither connexin 43 nor 45 was observed in NHOst cocultured with alumina microspheres, their GJIC was similar to that of normal NHOst. Moreover, it has reported that gap junctions formed by connexin 43 are more permeable to negatively charged dyes such as lucifer yellow, calcein, and carboxyfluorescein used in this study, more than those formed by

connexin 45, and an increase of connexin 43 expression and GJIC function parallel osteoblast differentiation.^{22,28} These are inconsistent with our findings and indicate that not high expression, but a rapid decrease of connexin 45 mRNA is probably very important for GJIC change and differentiation of the osteoblasts. Therefore, even though connexin 45 may play an important role in the early stage of GJIC in NHOst, it is probable that another connexins or other mechanisms of GJIC play a role in the GJIC of NHOst.

Because many proteins are involved in GJIC formation,²⁸ other mechanisms or proteins may be important in the GJIC change induced by the contact with the microspheres. It has reported that cadherins, which are important proteins for form tight junction between cells, control connexin 43-mediated GJIC.^{29,30} In addition, a microtubule network inside a cell has been reported to play an important role as guidance for delivery of connexons, which are composed of six connexin molecules, to the cell membrane to make gap junctions.³¹ Usually, surface characteristics of materials affect cell attachment as well as cell morphology, suggesting signal cascades of cell attachment and cytoskeleton rearrangement in the cell were influenced by the characteristics. Therefore, it is probable that a surface characteristic of the microspheres affect these molecules in NHOst, resulting in changes of GJIC activities. Further studies on changes in not only connexin molecules but also other molecules such as cadherin, actin, and microtubule in NHOst, is necessary to clarify the mechanism of GJIC. In the future, we will study the above, and find another molecules participating in the GJIC of NHOst and the mechanisms regulating the connexins in NHOst.

In conclusion, the GJIC level of NHOst changes on contact with microspheres, and is affected by the composition of the microspheres. The GJIC level in the early stage might be important in the differentiation control of NHOst and the level may be controlled partly by expression of connexin 43, connexin 45, and unclarified connexins in addition to other mechanisms regulating GJIC function. Detecting a biomaterial's effect on the GJIC of human cells may be one useful method for estimating its biocompatibility.

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