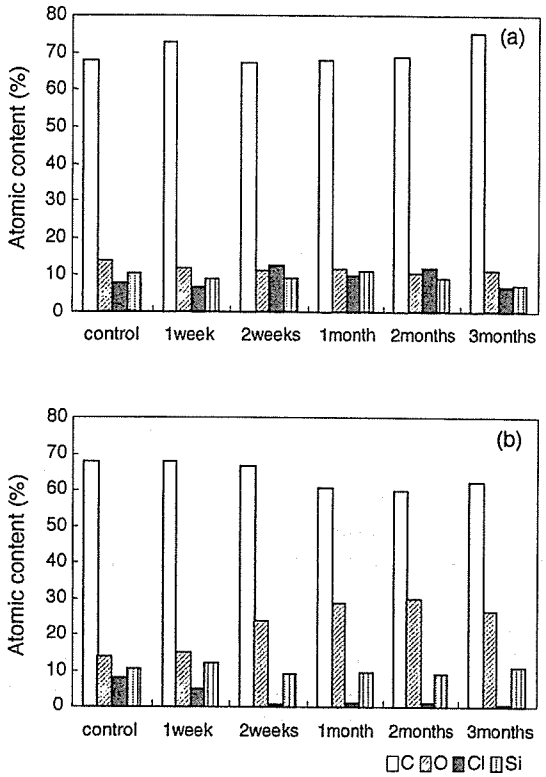

Fig. 4. Elemental analysis of PVC sheets by ESCA: (a) typical bar graph of PVC sheet samples subjected to heat treatment or visible light irradiation, and negative control and (b) bar graph of PVC sheet sample subjected to UV irradiation.

ence in the contact angle is due to differences in the embossed processing of the outer and inner surfaces (Fig. 5c (control) and Fig. 5b (control)). As shown in Fig. 5, the static angle of contact of the PVC sheets using the Sandimmun® injection as the wetting agent was not affected by either heat treatment or visible light irradiation (Fig. 5a). On the other hand, the static angle of contact of the inner surface of the UV-irradiated PVC sheets was decreased with time (Fig. 5b). In addition, the static angle of contact of the inner surface of the UV-irradiated positive control PVC sheets was almost the same as that of the inner surface of the PVC sheets UV-irradiated for 3 months. On the other hand, the static angle of contact of the outer surface of the UV-irradiated PVC sheets was increased markedly from the control to 3 months (Fig. 5c).

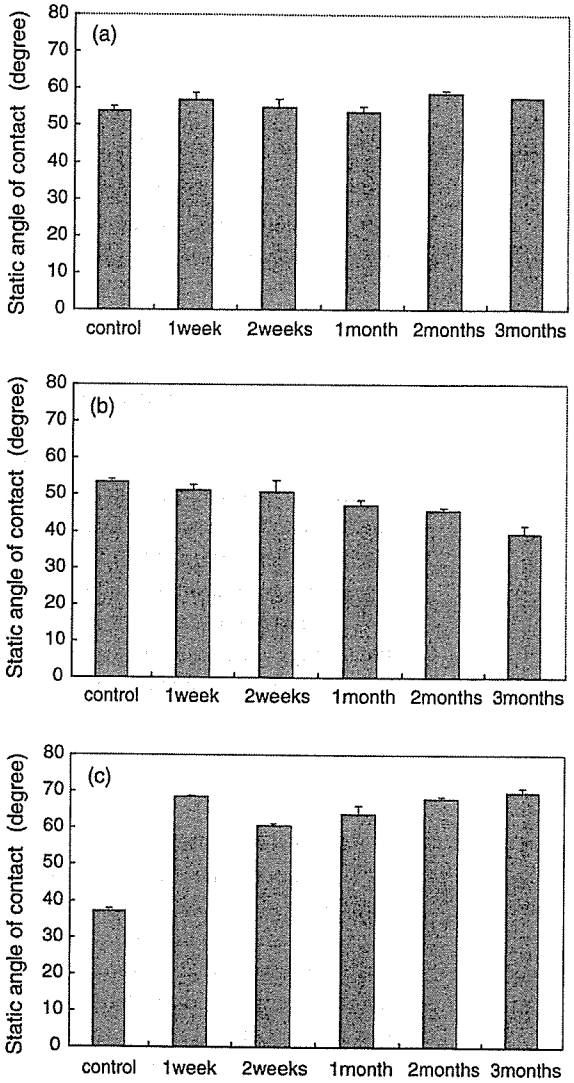

Fig. 5. Static angle of contact of PVC sheet samples Sandimmun® injection was used to determine the static angle of contact of PVC sheets subjected to heat treatment or optical irraditation. Typical bar graph of PVC sheet samples subjected to heat treatment or visible light irradiation. Bar graph of PVC sheet samples subjected to UV irradiation. The static angle of contact obtained by the inner surface of PVC sheet. Bar graph of PVC sheet samples subjected to UV irradiation. The static angle of contact obtained by the outer surface of PVC sheet.

Therefore, surface structure of the heat-treated PVC sheets and visible light irradiated PVC sheets did not change with time. On the other hand, surface structure of the UV-irradiated PVC sheets was markedly changed.

Table 2
Maximum force to break the PVC sheets for tensile test

	4 °C	37 °C	60 °C	Visible light	UV light
1 week	37.03 ± 1.68	36.54 ± 1.15	37.64 ± 2.01	36.63 ± 1.32	37.59 ± 0.86
2 weeks	36.12 ± 1.34	36.92 ± 0.52	36.20 ± 0.80	36.97 ± 1.12	36.28 ± 0.67
1 month	36.12 ± 1.07	36.86 ± 2.13	36.46 ± 1.39	36.70 ± 1.69	35.73 ± 0.76
2 months	37.84 ± 1.93	36.43 ± 2.14	36.08 ± 1.56	37.03 ± 0.39	36.43 ± 0.52
3 months	36.76 ± 1.48	36.28 ± 2.04	36.52 ± 0.81	36.86 ± 1.77	36.76 ± 1.05

($n = 4$) Negative control samples: 36.37 ± 0.78 N; positive control samples subjected to heat treatment: 37.28 ± 0.92 N; positive control samples irradiated with visible light: 37.11 ± 1.33 N; positive control samples irradiated with UV: 33.07 ± 2.88 N.

3.4. Tensile test

Flexibility and stability are some of the reasons why PVC products are used widely. In order to examine the deterioration of PVC products subjected to heat treatment or optical irradiation, the tensile strength and elasticity were measured. The maximum force to break the PVC sheets ranged from 33.1 ± 2.9 to 37.8 ± 1.9 N regardless of treatment (Table 2).

Therefore, there were no notable changes in the tensile strength and elasticity of the PVC sheets when heat treatment or optical irradiation was applied.

4. Conclusions

The DEHP content and the surface structure of the PVC products, and the levels of DEHP migrating from them were measured in order to determine the influence of external factors on PVC products during storage. In addition, a tensile test was carried out to determine the tensile strength and elasticity of the PVC products. It was hypothesized that UV irradiation led to changes in the surface structure, and that change was responsible for the decreased levels of DEHP migrating from the PVC products using a drug solvent diluted according to the package insert.

In order to clarify the change in the surface structure, we examined the surface by ESCA. In UV-irradiated PVC sheets, we observed that the hydrogen chloride level was decreased and oxidation proceeded with time. Similarly, in the FT-IR spectra, we observed that the absorption band characteristic of C–Cl stretching vibration from PVC and the C–H band from the aromatic compound were decreased with time. In addition, the absorption bands in the FT-IR spectra were found to broaden with time. PVC oxidation and crosslinking were surmised to explain these events. Based on these results, the UV irradiation of PVC products induced a decrease in the levels of DEHP migration, and the PVC products maintained their features, such as flexibility and stability. Some studies have reported the decreased DEHP release from PVC products by modifying the surface structure of the products under various conditions such as UV-irradiation with sodium azide as an enhancer for absorbing UV-energy, gamma-ray irradiation, in an aqueous solution containing water-soluble compounds such as methacrylic acid, and gas plasma treatment under reduced pressure (Jayakrishnan et al., 1995; Krishnan et al., 1991). In comparison with these techniques, the simple UV-irradiation method described in this study seems to have a great advantage, because it can be performed easily under atmosphere conditions without reagents or special instruments.

Today, the medical device industry is searching for a substitute for DEHP as a plasticizer. Our results suggest that the levels of DEHP migrating from a PVC product can be reduced by easy surface treatment without changing the type of plasticizer. This could be useful method to develop novel PVC products, if other safety aspects are confirmed. Possible biological changes should not be ignored, since increased oxygen content on the surface could have an important impact on the activation of the clotting system and complements. A detailed investigation is in progress in our laboratory to develop novel PVC products.

Acknowledgement

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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 BioWhitteker 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 m*M* 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× 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 (Amarsham 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'GGATCCTCAAGTTCCCTCCT3'). 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 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'TAAGTAGGACAACAAGGAGGTCGTGACGACGC3'; product size 578-bp). All reactions included negative controls without cDNA.

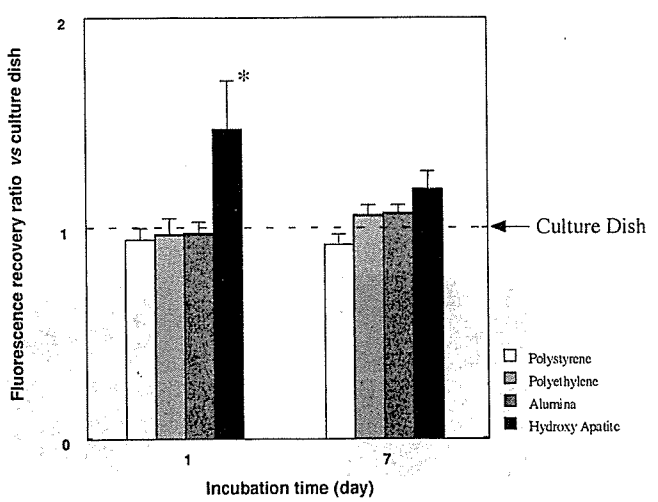

Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (*$p < 0.01$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

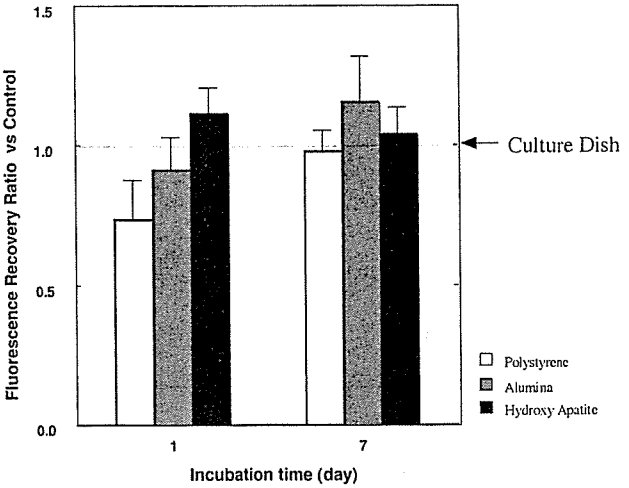

Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

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-

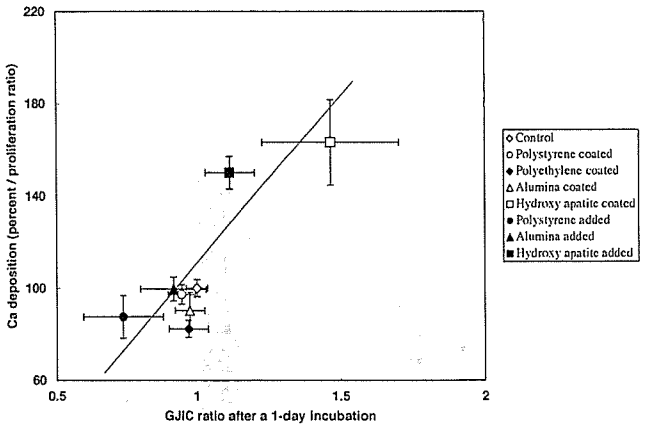

Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres (r^2 = 0.74).

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

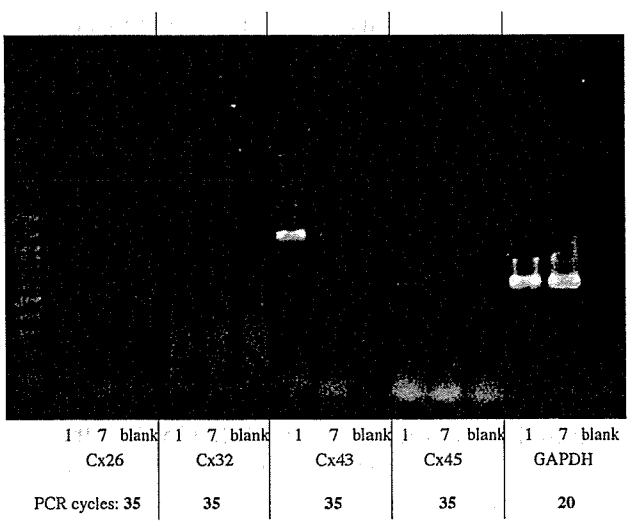

Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2 × 10^5. RT-PCR cycles of each lane are expressed at the bottom of the figure.

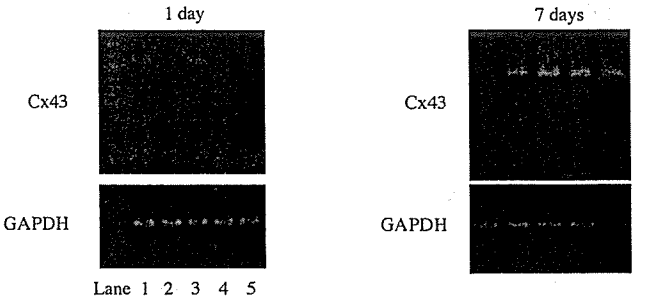

Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres;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

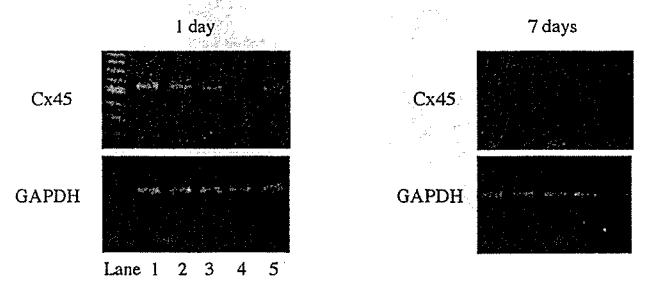

Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres;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

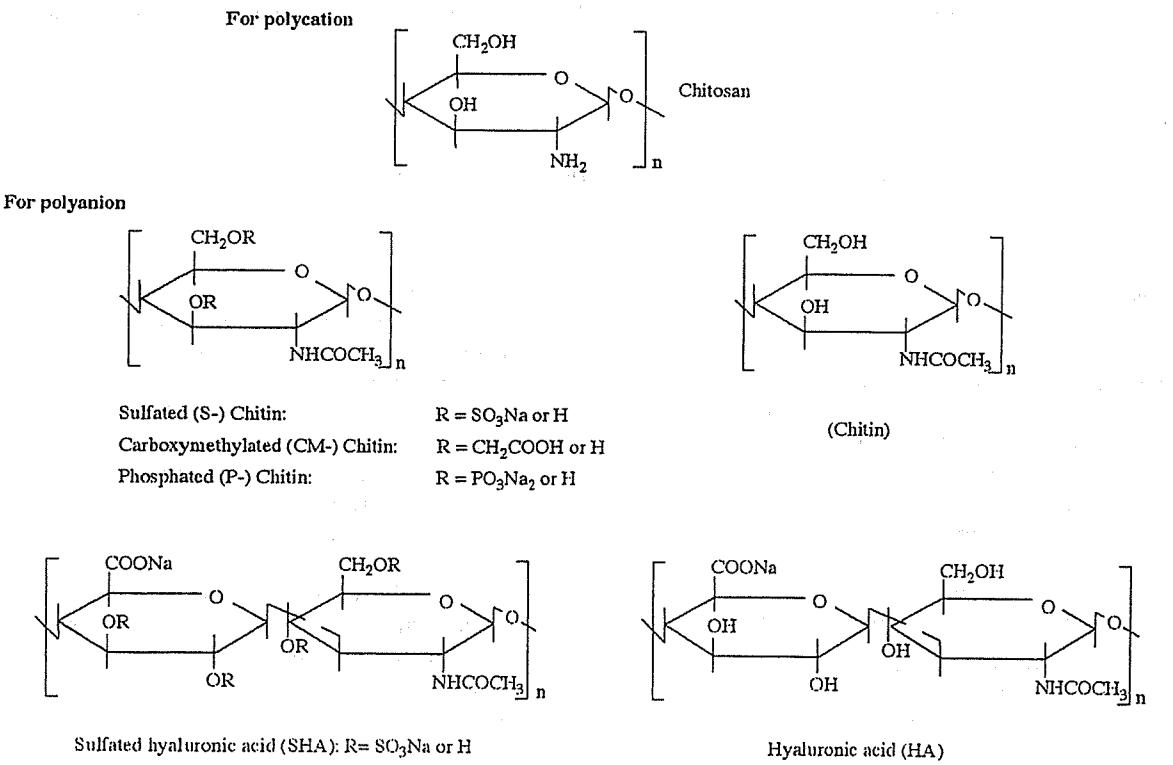

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

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.

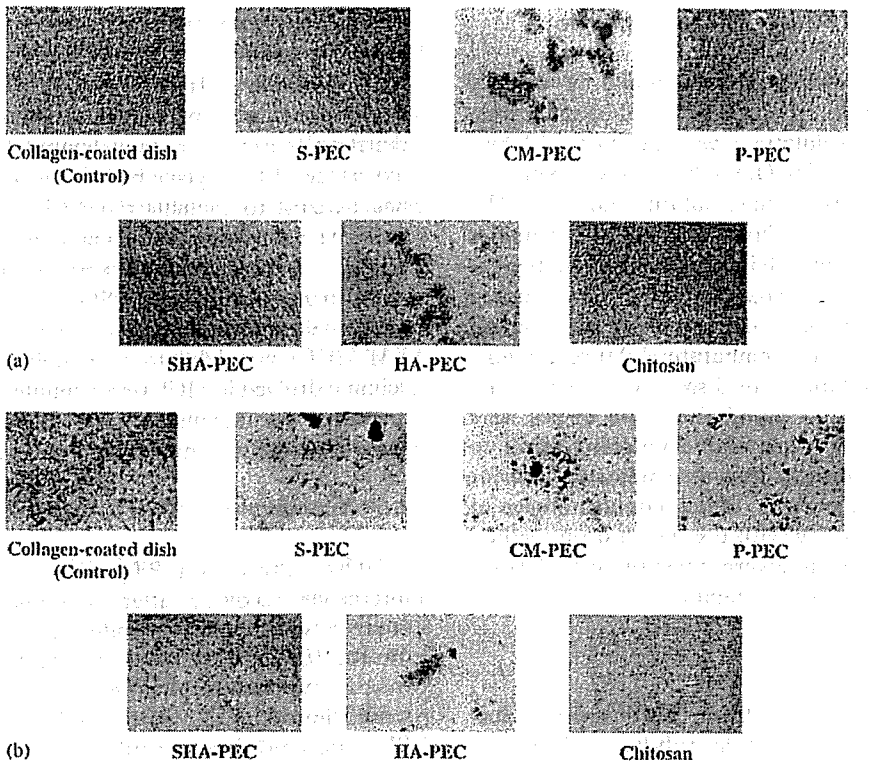

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

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

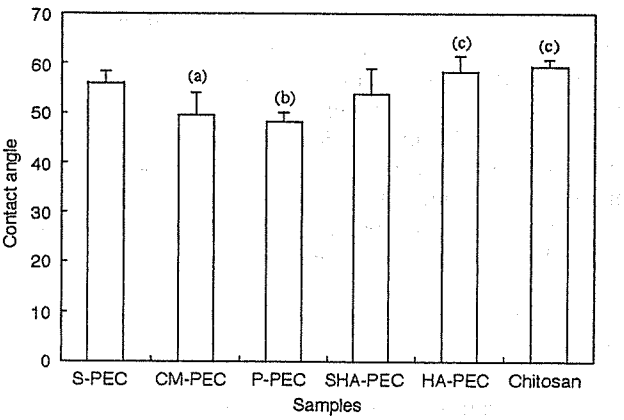

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

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

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

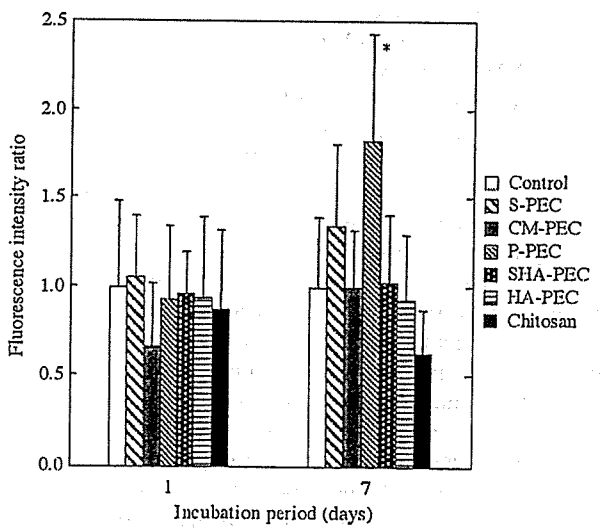

Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (*$p < 0.01$ against control).

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.

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Development of a simple method for predicting the levels of di(2-ethylhexyl) phthalate migrated from PVC medical devices into pharmaceutical solutions

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Abstract

This study deals with the development of a simple method for predicting the elution levels of di-2-ethylhexyl phthalate (DEHP) from medical devices made of polyvinyl chloride (PVC) by using the physicochemical properties of pharmaceutical injections as a marker. GC-MS analysis showed that the release of DEHP from medical grade PVC product was concentration-dependently increased by extraction with two kinds of lipophilic injections (Sandimmun® and Prograf®) and three kinds of surfactants (HCO-60, Tween® 80, and SDS). The solubility of lipophilic pigments such as Sudan III, methyl yellow, and 1,4-diamino-anthraquinone against these solutions were also increased in a concentration-dependent manner, in which methyl yellow showed the highest response regarding the increase of optical density (O.D.). Further, electrical conductivity and static contact angle to the PVC sheet of the solutions were also increased or decreased in the same manner. As a result of the comparative study, significant correlation was found between DEHP release levels and these three physicochemical properties, particularly methyl yellow solubility, of the solutions tested. To evaluate the relationship in detail, DEHP release levels from PVC tubing and methyl yellow solubility of 53 injections used in gynecologic and obstetric fields were determined. None of the hydrophilic medicines showed any significant release of DEHP, and all showed low solubility of methyl yellow. On the other hand, the lipophilic medicines releasing a large amount of DEHP showed high solubility of methyl yellow (greater than O.D. 0.8). These

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results indicate that a significant proportional relationship exists between DEHP release potency and methyl yellow solubility of pharmaceutical solutions, and the risk of DEHP exposure to the patients administered pharmaceuticals through transfusion set could be easily predicted by the solubility test without complicated elution tests of DEHP using GC-MS or LC-MS.

Keywords: DEHP; PVC; Medical device; Prediction; Risk assessment

1. Introduction

Phthalate esters, and DEHP in particular, have been extensively used as plasticizers due to the increased flexibility of PVC a plastic polymer used in a wide array of products including medical devices such as tubings, intravenous bags, blood containers, and catheters. DEHP is easily eluted from PVC products into not only foods but also pharmaceuticals and body fluids that come in contact with the plastic, and the migrated DEHP is directly and/or indirectly introduced into the human body (Allwod, 1986; Loff et al., 2000; Tickner et al., 2001). Some phthalates including DEHP are considered to be a toxic compound exhibiting effects similar to those of endocrine disruptors in rodents; they have antiandrogenic effects in male rats during the development of the male reproductive system and the production of normal sperm (Poon et al., 1997; Lamb et al., 1987; Tyl et al., 1988), and decrease the 17β-estradiol level in blood in female rats (Davis et al., 1994). General toxicity of DEHP has been well evaluated, and so far the result of risk assessment to human health indicates that this compound is relatively safe to humans. However, because the reproductive and developmental toxicity of DEHP to the human body is not well understood, it has recently been suggested that precautions be taken to limit the exposure of humans, particularly that of high risk patient groups such as male neonates, male fetuses, and peripubertal males, to DEHP. The concern is that DEHP's potency might have adverse effects on humans similar to those demonstrated on young rodents.

Taking the above into consideration, several agencies and official organizations in the world individually evaluated the safety of DEHP released from PVC products (Center for Devices and Radiological Health, 2001; Health Canada, 2002), and the Japanese Ministry of Health, Labor and Welfare (JMHLW) restricted the oral tolerable daily intake (TDI) value to 40–140 μg/kg/day.

It is very important that the exposure amount be exactly determined to conduct a risk assessment of the effect of DEHP on human health. Although some studies on the elution of DEHP from PVC medical devices have been performed as one of the JMHLW projects (Haishima et al., 2004; Inoue et al., 2003a,b; Takatori et al., 2004), it is not easy to identify the release behavior of DEHP from the variety of PVC products used in Japan by elution test under conditions that are the same as or similar to those of medical use. In addition, analytical methods having high sensitivity, precision, selectivity of quantitative ions, and low background, such as tandem LC-MS, high resolution GC-MS, and column-switching LC-MS methods, are required to determine DEHP for clinical assessment. Thus, regardless whether an investigation is in vivo or in vitro, the release test of DEHP is at present time-consuming and labor-intensive.

Jenke (2001) reported that the chemical compatibility assessment considers two distinct yet complementary mechanisms by which a device and its contacted solution can interact. These mechanisms include the migration of a chemical component out of the device and into the contacted solution (leaching) and the sorption of contained solution components by the device (binding). Alternatively, the product/device interaction can be modeled based on a rigorous scientific assessment of the physicochemical processes. Such models are based on the linear correlation of polymer/solution interaction constants with solvent/water partition coefficients (Nasim et al., 1972; Pitt et al., 1988; Hayward et al., 1990; Kenley and Jenke, 1990; Jenke, 1991; Jenke et al., 1991; Atkinson and Duffull, 1991; Roberts et al., 1991; Jenke et al., 1992). In addition, it is known that extraction occurs either by leaching or after an extracting material such as blood and pharmaceutical solutions diffuses into the PVC matrix and dissolves the plasticizer, which is relatively lipophilic. In consideration of these issues, we suspected that the release behavior of DEHP from PVC medical devices may be predicted from the physicochemical properties of

pharmaceutical injections applied to the devices, without a complicated elution test.

In the present study, to develop a simple method for predicting the release level of DEHP from PVC medical devices, we examined the relationship between the release potency of DEHP from PVC product and physicochemical properties such as the solubility of lipophilic pigments, electrical conductivity, and the static contact angle to PVC sheet, using two kinds of lipophilic injections and three kinds of surfactants as test solutions. Further, to evaluate the relationship in detail, DEHP release levels from PVC tubing and the physicochemical properties of 53 injections used in gynecologic and obstetric fields were determined.

2. Materials and methods

2.1. Chemicals and utensils

Medical grade PVC sheet for blood container and PVC tubing for transfusion set were provided by Terumo Co. (Tokyo, Japan).

Sandimmun® (50 mg/ml cyclosporine) and Prograf® (5 mg/ml tacrolimus) were provided by Novartis Pharma K.K. (Tokyo, Japan) and Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan). The other 51 injections listed in Table 1 were purchased from commercial companies. Polyoxyethene hydrogenated castor oil 60 (HCO-60) provided by Nikko Chemicals Co. (Tokyo, Japan), polysorbate 80 (Tween® 80, ICN Biomedicals Inc., Ohio, USA), and sodium lauryl sulfate (SDS, Sigma Aldrich Japan, Tokyo, Japan) were used as surfactants. In these materials, Sandimmun®, Prograf®, HCO-60, Tween® 80, and SDS were used as pretest solutions for evaluating the relationship between release potency of DEHP and physicochemical properties of pharmaceuticals.

Methyl yellow (Wako Pure Chemical Industries, Ltd., Osaka, Japan), Sudan III (Sigma Aldrich Japan, Tokyo, Japan), and 1,4-diamino-anthraquinone (Tokyo Kasei Co., Tokyo, Japan) were used as lipophilic pigments. DEHP and DEHP-d_4 were purchased from Kanto Chemical Co. (Tokyo, Japan). Hexane, anhydrous sodium sulfate, sodium chloride of phthalate esters of analytical grade, diethyl ether of dioxin of analytical grade, and distilled water of HPLC grade were used in this study.

All utensils were made of glass, metal, or teflon, and were heated at 250 °C for more than 16 h before use.

2.2. Classification of pharmaceuticals

As shown in Table 1, based on the properties of principal drugs and additives contained in each pharmaceutical, 53 injections used in this study were divided into five groups. Expression rule on solubility of the drugs has been established in general notices in the Japanese Pharmacopoeia IX edition regarding the relationship between descriptive term and the degree of dissolution. Pharmaceuticals such as Sandimmun® and Prograf® containing principal drugs that are expressed as practically insoluble or insoluble to water in the instruction manuals were assigned to group 1 as lipophilic injections. Most of pharmaceuticals in this group were contained various additives such as surfactants, oils, glycerin, ethanol, benzyl alcohol, and so on. The principal drugs of pharmaceuticals classified into group 2 are also insoluble or very slightly soluble to water, but these drugs can be dissolved in acidic or basic solutions. Gaster®, Droleptan®, Elaspol®, Aleviatin®, Methotrexate® Parenteral, Serenace®, and Bosmin® were assigned to this group, and pH of each pharmaceutical is expressed in the instruction manuals as 4.7–5.7, 2.5–4.5, 7.5–8.5, approximately 12, 7.0–9.0, 3.5–4.2, and 2.3–5.0, respectively. Pharmaceuticals consisted of drugs that are slightly soluble or sparingly soluble to water were classified into group 3. Solubility of principal drugs contained in the pharmaceuticals assigned to groups 4 and 5 was expressed as very soluble, freely soluble, or soluble to water in each instruction manual. Pharmaceuticals of group 5 are hydrophilic injections as negative control regarding DEHP migration. Although pharmaceuticals assigned to group 4 are also hydrophilic injections, these pharmaceuticals were suspected to induce DEHP migration, because some of them are human serum products or containing chlorobutanol, phenol, and benzyl alcohol as additives.

2.3. Solubility test of lipophilic pigments

One millilitre of each surfactant solution and pharmaceutical injection was added to each lipophilic pigment (5 mg) followed by sonication for 10 min at room temperature and centrifugation at 3000 rpm for 10 min. The supernatant was passed through a membrane filter (pore size 0.2 μm) and the filtrate (100 μl) was

Table 1
List of phamaceutical injections used in this study

Product name	Principal drug	Concentration for medical use	Additives	Medication	Color
Group 1[a]					
Sandimmun®	Cyclosporin	500 μg/mL	Polyoxyethene castor oil, ethanol	Instillation	Clear
Prograf® injection 5 mg	Tacrolimus hydrate	10 μg/mL	Absolute ethanol, HCO-60	Instillation	Clear
1% Diprivan® injection	Propofol	10 mg/mL	Soybean oil, concentrated glycerin, pure egg-yolk lecithin, edetate sodium pH adjuster	Intravenous injection	White emulsion
Ropion®	Flurbiprofen axetil	10 mg/mL	Pure soybean oil, pure egg-yolk lecithin, concentrated glycerin	Intravenous injection	White emulsion
Sohvita®	Vitamins including fat-soluble vitamin	Whole amount of Sobita was mixed with PN-Twin No.2 (2.2 L)	Sodium citrate, pH adjuster, sodium pyrosulfite, sodium thioglycollate, HCO-60, benzyl alcohol, polysorbate 80	Instillation	Yellow (clear)
Kaytwo® N	Menatetrenone	5 mg/mL	Aminoethylsulfonic acid, sesame oil, pure soybean lecithin, D-sorbitol, concentrated glycerin, pH adjuster	Intravenous injection	Buff yellow (translucence)
Humulin® R	Insulin human	40 units/mL	Concentrated glycerin, *m*-cresol, pH adjuster	Intravenous injection	Clear
Prostarmon®-F	Dinoprost	2 mg/mL		Instillation	Clear
Florid®-F	Miconazole	1 mg/mL	HCO-60	Instillation	Clear
Horizon®	Diazepam	5 mg/mL	Propylene glycol, ethanol, benzyl alcohol, sodium benzoate, benzoic acid	Intravenous injection	Buff yellow (clear)
Predonine®	Prednisolone sodium succinate	① 10 mg/mL, ② 1 mg/mL	Dried sodium carbonate, sodium hydrogenphosphate, sodium dihydrogenphosphate crystal	① Intravenous injection, ② instillation	Clear
Group 2[a]					
Gaster®	Famotidine	20 mg/mL	L-Aspartic acid, D-mannitol	Instillation	Clear
Droleptan®	Droperidol	① 2.5 mg/mL, ② 50 μg/mL	*p*-Oxymethyl benzoate, *p*-oxypropyl benzoate pH adjuster (acidic)	① Intravenous injection, ② instillation	Clear
Elaspol®	Sivelestat sodium hydrate	1 mg/mL	D-Mannitol, pH adjuster	Intravenous injection	Clear
Aleviatin®	Phenytoin	50 mg/mL	Sodium hydroxide, propylene glycol, ethanol	Intravenous injection	Clear
Methotrexate® parenteral	Methotrexate	0.2 mg/mL	Sodium chloride, sodium hydroxide	Instillation	Clear
Serenace®	Haloperidol	5 mg/mL	Glucose, lactic acid, sodium hydroxide	Instillation	Clear
Bosmin® injection	Epinephrine	0.25 mg/mL	Chlorobutanol, sodium hydrogen sulfite, hydrochloric acid, sodium chloride, pH adjuster	Intravenous injection	Clear
Group 3[a]					
Partan M injection	Methylergometrine maleate	0.2 mg/mL		Intravenous injection	Clear
Musculax® intravenous	Vecuronium bromide	2 mg/mL	D-Mannitol	Intravenous injection	Clear
Carbenin® for intravenous drip infusion	Panipenem Betamipron	5 mg/mL	pH Adjuster	Instillation	Achroma yellow (clear)