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

Ryusuke Nakaoka, Saifuddin Ahmed, Toshie Tsuchiya
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 26 September 2004; revised 14 December 2004; accepted 14 December 2004
Published online 17 June 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30328

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
Correspondence to: R. Nakaoka; e-mail: nakoaka@nihs.go.jp

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.

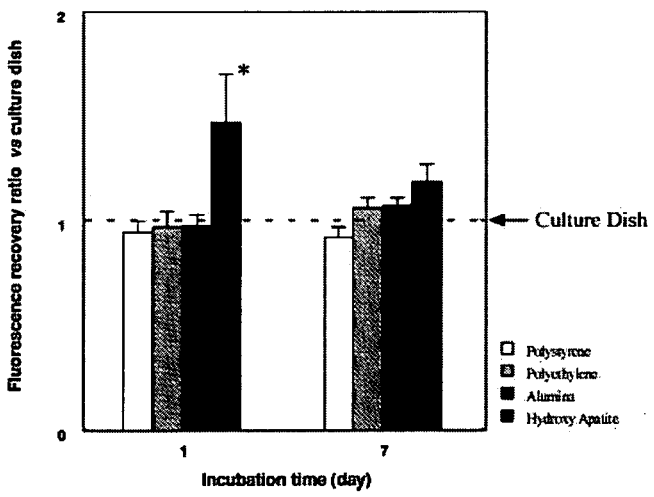

Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (*$p < 0.01$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

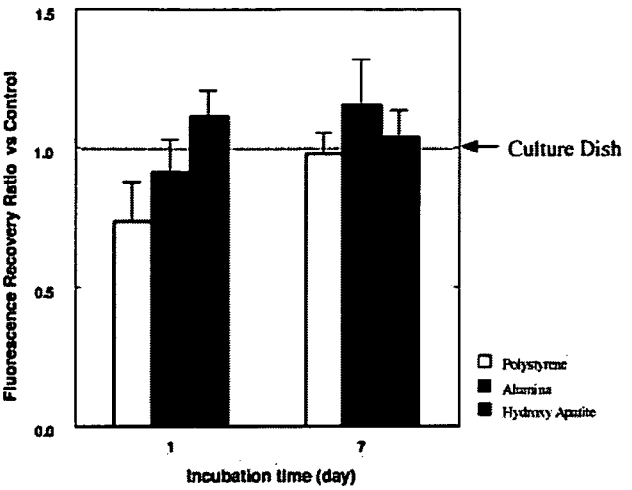

Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

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-

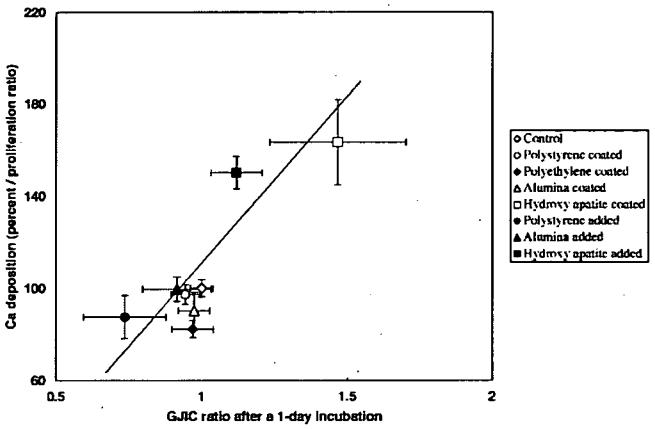

Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres (r^2 = 0.74).

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

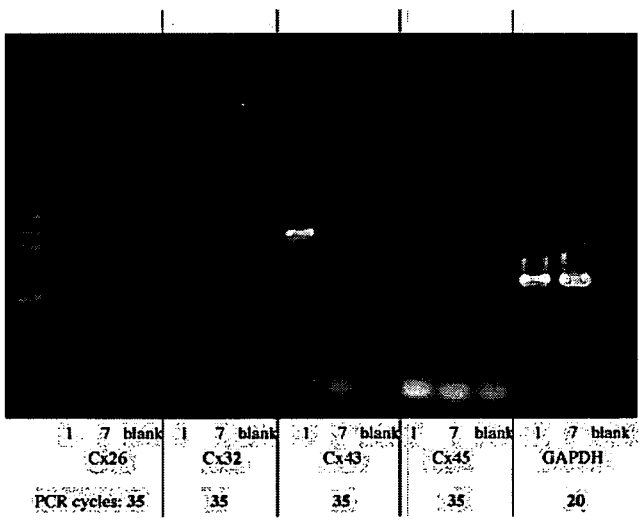

Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2 × 10^5. RT-PCR cycles of each lane are expressed at the bottom of the figure.

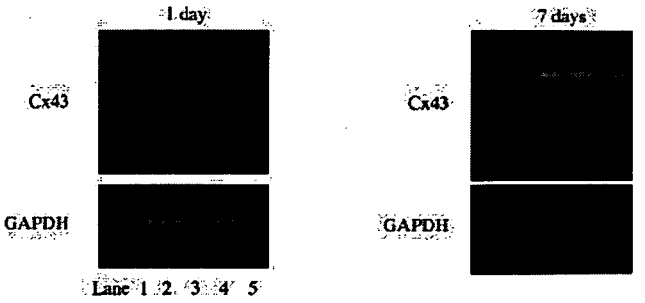

Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres;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

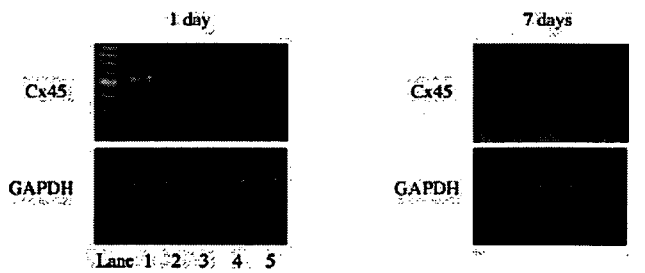

Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres;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

The authors appreciate the support of Health and Labor Sciences Research Grants for Research on Advanced Medical Technology, Research on Health Sciences focusing on Drug Innovation, and Risk Analysis Research on Food and Pharmaceuticals, Ministry of Health, Labour and Welfare.

References

1. Willert HG, Semlitsch M. Reactions of the articular capsule to wear products of artificial joint prosthesis. J Biomed Mater Res 1977;11:157–164.

2. Savio JA III, Overcamp LM, Black J. Size and shape of biomaterial wear debris. Clin Mater 1994;15:101–147.
3. Wang W, Ferguson DJP, Quinn JMW, Simpson AHRW, Athanasou NA. Osteoclasts are capable of particle phagocytosis and bone resorption. J Pathol 1997;182:92–98.
4. Goodman SB, Fornasier VL, Lee J, Kei J. The histological effects on the implantation of different sizes of polyethylene particles in the rabbit tibia. J Biomed Mater Res 1990;24:517–524.
5. Kubo T, Sawada K, Hirakawa K, Shimizu C, Takamatsu T, Hirasawa Y. Histiocyte reaction in rabbit femurs to UHMWPE, metal, and ceramic particles in different sizes. J Biomed Mater Res 1999;45:363–369.
6. Shanbhag AS, Jacobs JJ, Black J, Galante JO, Glant TT. Macrophages/particle interactions: Effect of size, composition and surface area. J Biomed Mater Res 1994;28:81–90.
7. Kim KJ, Itoh T, Tanahashi M, Kumegawa M. Activation of osteoclasts-mediated bone resorption by the supernatant from a rabbit synovial cell line in response to polyethylene particles. J Biomed Mater Res 1996;32:3–9.
8. Voronov I, Santerre JP, Hinek A, Callahan JW, Sandhu J, Boynton EL. Macrophage phagocytosis of polyethylene particles in vitro. J Biomed Mater Res 1998;39:40–51.
9. Catelas I, Huk OL, Petit A, Zukor DJ, Marchand R, Yahia L. Flow cytometric analysis of macrophage response to ceramic and polyethylene particles: Effects of size, concentration, and composition. J Biomed Mater Res 1998;41:600–607.
10. Green TR, Fisher J, Stone MH, Wroblewski BM, Ingham E. Polyethylene particles of a "critical size" are necessary for the induction of cytokines by macrophages in vitro. Biomaterials 1998;19:2297–2302.
11. Green TR, Fisher J, Matthews JB, Stone MH, Ingham E. Effect of size and dose on bone resorption activity of macrophages by in vitro clinically relevant ultra high molecular weight polyethylene particles. J Biomed Mater Res (Appl Biomater) 2000; 53:490–497.
12. Takei I, Takagi M, Santavirta S, Ida H, Hamasaki M, Ishii M, Fukushima S, Ogino T, Konttinen YT. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in joint fluid of the patients with loose artificial hip joints. J Biomed Mater Res 1999;45:175–183.
13. Trindade MCD, Schurman DJ, Maloney WJ, Goodman SB, Smith RL. G-protein activity requirement for polymethylmethacrylate and titanium particle-induced fibroblast interleukin-6 and monocyte chemoattractant protein-1 release in vitro. J Biomed Mater Res 2000;51:360–368.
14. Sacomen D, Smith RL, Song Y, Fornasier V, Goodman SB. Effects of polyethylene particles on tissue surrounding knee arthroplasties in rabbits. J Biomed Mater Res (Appl Biomater) 1998;43:123–130.
15. Tsuchiya T, Hata H, Nakamura A. Studies on the tumor-promoting activity of biomaterials: Inhibition of metabolic cooperation by polyetherurethane and silicone. J Biomed Mater Res 1995;29:113–119.
16. Nakaoka R, Tsuchiya T, Kato K, Ikada Y, Nakamura A. Studies on tumor-promoting activity of polyethylene: Inhibitory activity of metabolic cooperation on polyethylene surfaces is markedly decreased by surface modification with collagen but not with RGDS peptide. J Biomed Mater Res 1997;35:391–397.
17. Nakaoka R, Tsuchiya T, Nakamura A. The inhibitory mechanism of gap junctional intercellular communication induced by polyethylene and the restorative effects by surface modification with various proteins. J Biomed Mater Res 2001;57:567–574.
18. Nakaoka R, Sakaguchi K, Tsuchiya T, Nakamura A. Studies on in vitro evaluation for the biocompatibility of various biomaterials: Inhibitory activity of various kinds of polymer microspheres on metabolic cooperation. J Biomed Mater Res 2001; 57:279–284.
19. Mensil M, Krutovskikh V, Omori Y, Yamasaki H. Role of blocked gap junctional communication in non-genotoxic carcinogenesis. Toxicol Lett 1995;82/83:701–706.
20. Yamasaki H. Role of disrupted gap junctional intercellular communication in detection and characterization of carcinogens. Mutat Res 1996;365:91–105.
21. Sawada MS, Mano H, Hanada K, Kakudo S, Kameda T, Miyazawa K, Nakamaru Y, Yuasa S, Mori Y, Kumegawa M, Hakeda Y. Down-regulation of gap junctional intercellular communication between osteoblastic MC3T3–E1 cells by basic fibroblast growth factor and a phorbol ester (12-O-tetradecanoylphorbol-13-acetate). J Bone Miner Res 1997;12:1165–1173.
22. Lecanda F, Towler DA, Ziambaras K, Cheng SL, Koval M, Steinberg TH, Civitelli R. Gap junctional communication modulates gene expression in osteoblastic cells. Mol Biol Cell 1998; 9:2249–2258.
23. Nakaoka R, Tsuchiya T. Biocompatibility of various kinds of polymer microspheres estimated from their effect on gap junctional intercellular communication of fibroblasts. Mater Trans 2002;43:3122–3127.
24. Wade MH, Trosko JE, Steindler M. A fluorescence photobleaching assay of gap junctional-mediated communication between human cells. Science 1986;232:525–528.
25. Ichikawa R, Tsuchiya T. A strategy for the suppression of tumorigenesis induced by biomaterials: Restoration of transformed phenotype of polyetherurethane-induced tumor cells by Cx43 transfection. Cytotechnology 2002;39:1–8.
26. Donahue HJ, Li Z, Zhou Z, Yellowley CE. Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication. Am J Physiol Cell Physiol 2000;278:C315–C322.
27. Laing JG, Manley-Markowski RN, Koval M, Civitelli R, Steinberg TH. Connexin45 interacts with zonula occludens-1 and connexin43 in osteoblastic cells. J Biol Chem 2001;276:23051–23055.
28. Duffy SH, Delmar M, Spray DC. Formation of the gap junction nexus: Binding partners for connexins. J Pathol Paris 2002;96: 243–249.
29. Jongen WMF, Fitzgerald DJ, Asamoto M, Piccoli C, Slaga TJ, Gros D, Takeichi M, Yamasaki H. Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. J Cell Biol 1991;114:545–555.
30. Meyer RA, Laird DW, Revel JP, Johnson RG. Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. J Cell Biol 1992;119:179–189.
31. Lauf U, Giepmans BNG, Lopez P, Braconnot S, Chen SC, Falk MM. Dynamic trafficking and delivery of connexins to the plasma membrane and accretion to gap junctions in living cells. Proc Natl Acad Sci USA 2002;99:10446–10451.

Effects of a biodegradable polymer synthesized with inorganic tin on the chondrogenesis of human articular chondrocytes

Nasreen Banu, Toshie Tsuchiya, Rumi Sawada
Division of Medical Devices, National Institute of Health Sciences, 1–18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 8 September 2005; accepted 14 September 2005
Published online 14 December 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30616

Abstract: Recent study has shown that biodegradable polymers are attractive candidates for chondrocyte fixation and further transplantation in cartilage tissue engineering. Poly (glycolic acid) (PGA), a polymer of glycolic acid, is widely used in orthopedic applications as a biodegradable polymer. Organotin, lead, antimony, and zinc are catalysts commonly used in synthesizing PGA. Here, we investigated the biocompatibility of PGA, synthesized with and without inorganic tin as a catalyst in chondrogenesis of human articular chondrocytes in a micromass culture system. Significant enhancement of chondrocyte proliferation and expression of the collagen type II protein gene were observed in cultures treated with PGA synthesized with a tin catalyst. However, aggrecan gene expression was very similar to the control culture. Amount of collagen type II protein was also increased in the same group of cultured chondrocytes. In contrast, PGA without a catalyst caused overall inhibition of chondrogenesis. Despite several positive findings, extensive investigations are essential for the feasibility of this PGA(Sn) in future clinical practice. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 84–89, 2006

Key words: poly (glycolic acid); inorganic tin catalyst; human articular cartilage; chondrogenesis; micromass culture

INTRODUCTION

Different synthetic biodegradable polymers are currently gaining importance in the fields of biotechnology and tissue engineering. Recently, many studies have evaluated the potential of various natural bioabsorbable polymers such as collagen, [1,2] alginates, [3–5] fibrin, [6–8] and gelatin, [9] but synthetic biodegradable polymers in general offer advantages over natural materials. The primary advantages include the capacity to change the mechanical properties and degradation kinetics to suit various applications. Among the families of synthetic polymers, polyesters are used in a number of clinical applications.[10–12] Polyesters have also been used for development of tissue engineering applications, [13,14] particularly for bone tissue engineering.[15,12]

Correspondence to: T. Tsuchiya; e-mail: tsuchiya@nihs.go.jp
Contract grant sponsor: Health and Labour Sciences Research
Contract grant sponsor: Ministry of Health, Labour and Welfare (Japan)
Contract grant sponsor: Japan Health Sciences Foundation

The attraction of poly (glycolic acid) (PGA), one of the aliphatic polyesters, as a biodegradable polymer in medical applications is that its degradation product, glycolic acid, is a natural metabolite. Several studies have indicated that copolymers of glycolic acid caused promotion of nerve regeneration in a rat model, [16–18] and regeneration of an 80 mm nerve gap by an artificial nerve conduit made of PGA was also reported.[19] PGA can be synthesized using different catalysts. The common catalysts used include organotin, lead, antimony, and zinc. It was reported that inorganic and organic tin compounds present in the aqueous ecosystem have toxic effects and are capable of producing behavioral abnormalities in living organisms.[20,21] Organotin compounds are known to cause neurotoxicity, [22] cytotoxicity, [23] immunotoxicity, and genotoxicity [24] in human and other mammalian cells both *in vitro* and *in vivo*. Organotin compounds were also reported to decrease *in vitro* survival, proliferation, and differentiation of normal human B cells.[25] The dose effect of inorganic tin in rats suggests that the critical organ in inorganic tin toxicity is bone, [26] and disproportionate dwarfing syndrome, which severely affects the limbs but not the trunk, was observed in rats that had been injected with certain tin compounds.[27] As far as we know, no study yet has reported the chondrogenic

effects of PGA synthesized with and without an inorganic tin catalyst. In this study, the biocompatibility of PGA with and without a tin catalyst was investigated, using human articular chondrocytes (HAC) in a micromass culture system.

MATERIALS AND METHODS

Medium and polymers used for cell culture

Chondrocyte growth medium was obtained commercially from BioWhittaker (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (M_w = 1500) and without a catalyst (PGA) (M_w = 1100) were custom-made (TAKI chemicals, Kakogawa, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA).

Cells and culture methods

Human articular chondrocytes (HAC) of the knee joint was commercially obtained from BioWhittaker. High-density micromass cultures were started by spotting 4×10^5 cells in 20 μL of medium onto Costar 24-well tissue culture microplates (Costar type 3526, Corning). After a 2 h attachment period at 37°C in a CO_2 incubator, culture medium (1 mL/well) was added to each well. Media were supplemented with DMSO (0.8 μL/mL), PGA, and PGA(Sn) (50 μg/mL). HAC cultured with DMSO was used as the control. The cultures were continued for 4 weeks with a medium change twice a week. At least four cultures were performed for each sample.

Cell proliferation study

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[28] After the culture period, cells were fixed with 100% methanol at room temperature, followed by application of 0.1% crystal violet in methanol. After a proper wash, cells were again incubated in methanol; 100 μL from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm, using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

Cell differentiation assay was performed by alcian blue (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[29] Following crystal violet staining, the cells were washed with methanol and then 3% acetic acid. Cultures were then stained with 1% (v/v) alcian blue in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4*M* guanidine hydrochloride (GH), and the bound dye was measured at wavelength of 600 nm, using an ELISA reader (Bio-Tek Instruments). Fresh 4*M* GH served as the blank. Blank values were subtracted from experimental values to eliminate background readings.

Analytical assays

Commercially available assay kits (collagen and glycosaminoglycan [GAG] assay kits, Biocolor, Newtownabbey, Northern Ireland) were used for the measurement of collagen and sulfated GAGs within the cultured cells, as previously described.[30]

Briefly, for the GAG assay, GAG was extracted from the cultured cells using a solvent system of 4*M* guanidine-HCl, 0.5*M* sodium acetate, pH 6, with 1 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* *N*-ethylmaleimide (NEM). Incubation was carried out at 4°C on an orbital shaker for a 12- to 20-h period. After the extraction, the samples were centrifuged, and blyscan dye reagent (composed of 1,9-dimethyl methylene blue in an organic buffer) was mixed with the supernatant. The GAG–dye complex was collected by centrifugation. The dye bound to the pellet was subsequently solubilized by mixing it with a dissociation reagent. The absorbance of the samples was measured at a wavelength of 656 nm, using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen concentration (acid- and pepsin-soluble fractions) of the cultured chondrocytes was also measured. The acid-soluble collagen was removed by adding 0.5*M* acetic acid to the cultured cells, followed by centrifugation. The remaining pepsin-soluble collagen was subsequently extracted from the cultured cells. A pepsin solution (1 mg/10 mg tissue sample; Sigma) was added to the cells, and they were incubated overnight at 37°C. Both the acid- and pepsin-soluble collagen samples were further separated for assay by mixing with Sircol dye reagent for 30 min in a mechanical shaker, and the collagen–dye complex was collected by centrifugation. The dye bound to the collagen pellet was solubilized with an alkaline reagent, and the absorbance of the samples was measured at a wavelength of 540 nm, using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment.

Real-time polymerase chain reaction

To detect the presence of collagen type II and aggrecan, single-stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT), using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently, real-time polymerase chain reaction (PCR) was done using a LightCycler system with LightCycler FastStart DNA Master SYBR Green I

(Roche Diagnostics, Penzberg, Germany). The LightCycler™- Primer set (Roche Diagnostics) was used for quantitative detection of the collagen type II and aggrecan genes, and also for quantitation of a housekeeping gene, Glyceraldehide-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's instructions. An initial denaturation step at 95°C for 10 min was followed by amplification and extension steps for 35 cycles (95°C for 10 s, 68°C for 10 s, 72°C for 16 s) with final extension step at 58°C for 10 s. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Statistical study

Student's t tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation) except in figure 3. Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell proliferation

Chondrocyte proliferation was quantified by crystal violet staining and expressed as a percentage of the average control value (Fig. 1). Cell proliferation was increased 1.8-fold ($p < 0.05$) in PGA(Sn)-treated cultures compared with that of the control culture, whereas cell proliferation in PGA-treated cultures was almost identical to the DMSO-treated control culture.

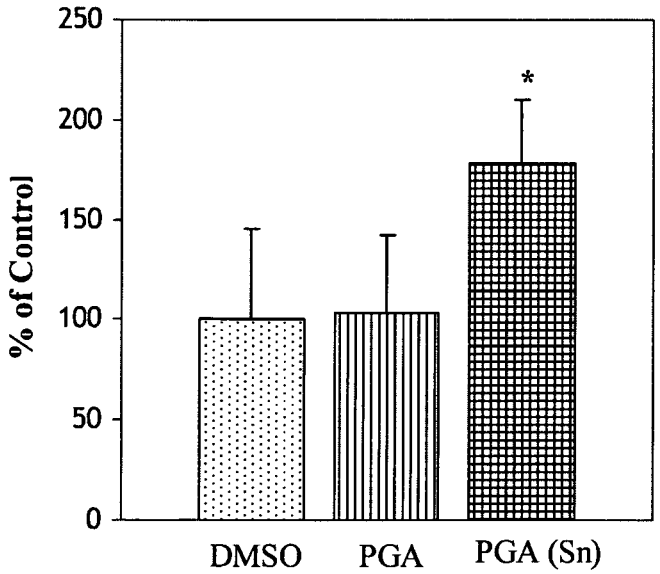

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

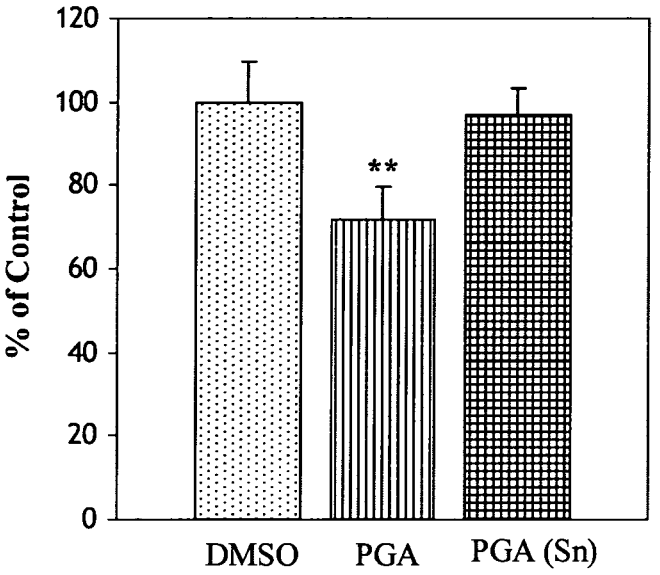

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

Chondrocyte differentiation was estimated by alcian blue staining and the amounts were expressed as a percentage of the average control value, which was calculated as 100%. Chondrocytes treated with PGA revealed a 0.71-fold ($p < 0.01$) decrease in cell differentiation compared with that of the control culture. At the same time, cultures treated with PGA(Sn) showed a slight, but nonsignificant, decrease in cell differentiation (Fig. 2).

Extracellular matrix gene expression

Extracellular matrix gene expression was quantitatively measured by real-time PCR. Here, compared with that of the control culture, the collagen type II gene was more strongly expressed ($p < 0.01$) in PGA(Sn) than in PGA-treated cultured chondrocytes [Fig. 3(A)]. Aggrecan gene expression was inhibited in the latter, but no difference was observed between the former and the control culture [Fig. 3(B)].

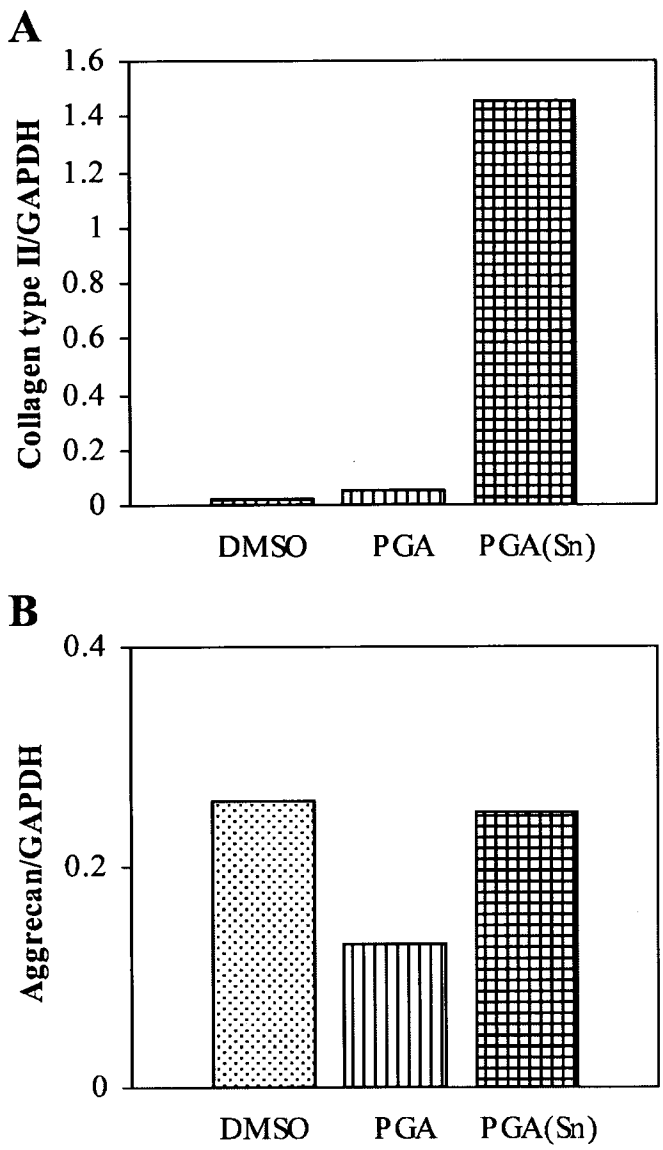

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between the PGA(Sn) and the control. All experiments were run in quadruplicate for two separate times.

Measurement of collagen type II protein

The amount of pepsin-soluble and cartilage-specific collagen type II protein was increased in both PGA and PGA(Sn) treated chondrocytes on comparing with that of the control culture(Fig. 4). However, this increase was more in the latter than in the former case.

Measurement of total collagen

Quantitative estimations of both acid- and pepsin-soluble total collagen revealed a decrease in PGA(Sn)-treated cultures compared with that of the control

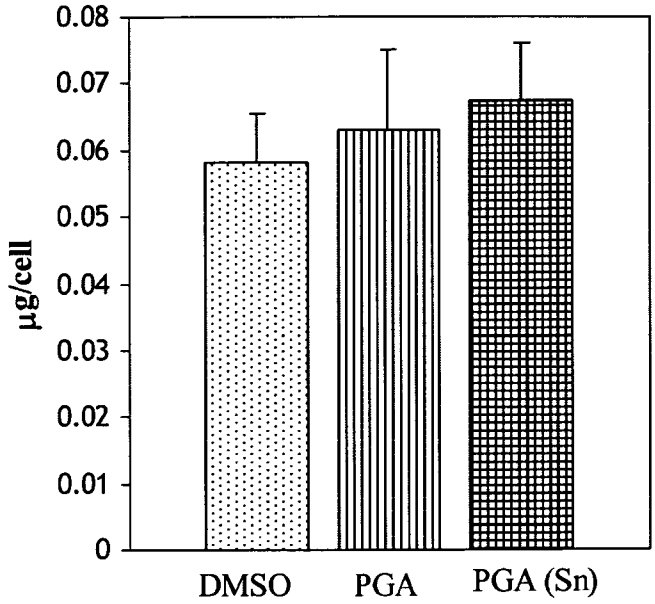

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(Fig. 5). Simultaneously, there was a slight increase in the amount of total collagen in PGA-treated cultures compared with that of the control sample.

Estimation of sulfated glycosaminoglycan concentration

Evaluation of the amount of sulfated GAG showed a decrease in PGA(Sn)-treated cultured cells com-

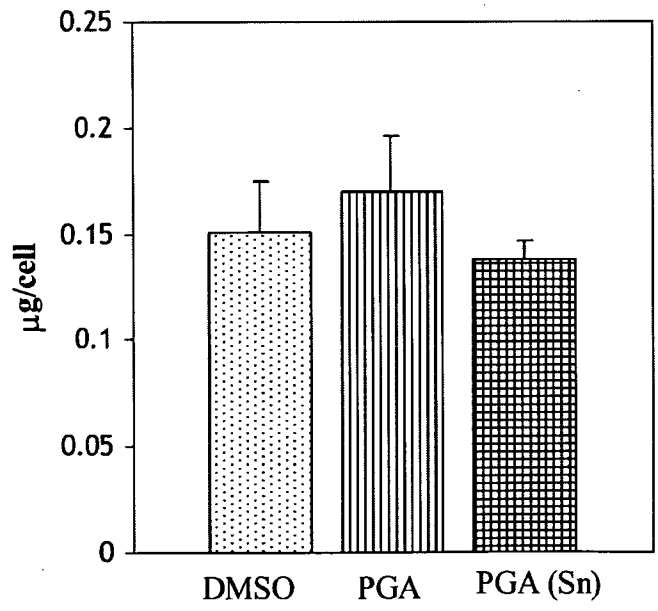

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

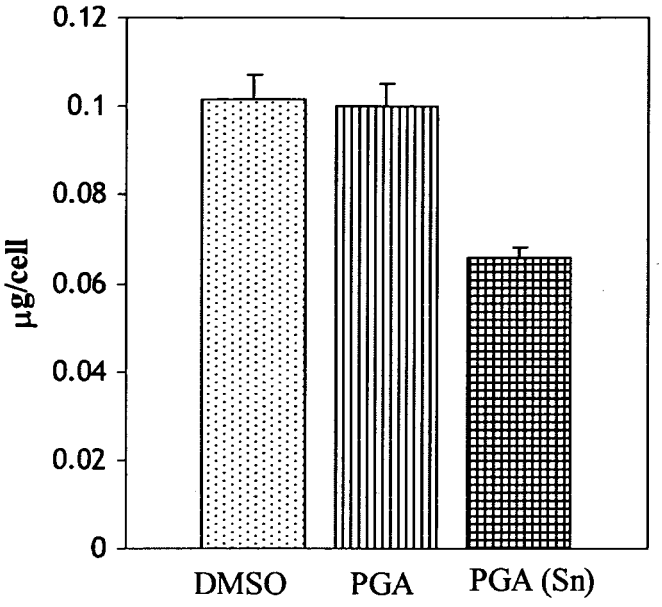

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

pared with that of the control (Fig. 6). However, in the same experiment, almost no difference in this amount was observed between the PGA-treated culture and the control.

DISCUSSION

Attempts to identify a perfectly biocompatible and biodegradable polymer have been ongoing over the past decade. An ideal biomaterial should fulfill its purpose satisfactorily and then biodegrade to obviate any risk of foreign body reaction.[31] Synthetic biodegradable polymers, especially those belonging to the polyester family, have played an important role in a number of tissue engineering efforts. PGA, an aliphatic polyester, can be degraded in two ways: by hydrolysis and by nonspecific esterases and carboxypeptidases, followed by either excretion in the urine or entrance into the tricarboxylic acid cycle.[32]

Several different catalysts, namely organotin, antimony, zinc, and lead, are used in the polymerization process to synthesize high molecular weight PGA. Different tin compounds were observed to produce general cytotoxic effects in rabbit articular cartilage in monolayer culture, [33] and bone is suggested to be the critical organ in inorganic tin toxicity in rats.[26] Therefore, in this study, we aspired to evaluate the chondrogenic effects of HAC with PGA synthesized with and without an inorganic tin catalyst, with the aim of clarifying the biocompatibility of inorganic tin as a catalyst for future clinical use.

It was reported that oral administration of certain tin compounds at specific concentrations exerted stimulatory effects on chondrocyte proliferation in the rat.[33] Consistent with this, the proliferation assay performed in our study also showed that HAC with PGA(Sn) had stimulatory effects on chondrocyte proliferation in micromass culture (Fig. 1). On the other hand, PGA neither stimulated nor inhibited the chondrocyte proliferation, and thus, inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In our experiment, PGA with inorganic tin as the catalyst caused almost no change in cell differentiation, but PGA-treated cultures did show a significant decrease when compared with that of the control (Fig. 2). Furthermore, quantitative estimation of extracellular matrix gene expression by real-time PCR confirmed that the cartilage-specific protein, collagen type II, was more strongly expressed in PGA(Sn)- than in PGA-treated cultured chondrocytes [Fig. 3(A)]. However, the expression of the aggrecan gene was inhibited in the PGA culture, but no difference was observed between the PGA(Sn) and the control cultures [Fig. 3(B)].

It was reported that oral administration of inorganic tin caused a decrease in the proliferation of chondrocytes, accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis in rat.[34] On the contrary, our results showed enhancement of proliferation, expression of the collagen type II gene, and amount of collagen type II protein by *in vitro* culture of HAC with PGA(Sn). We speculated that difference in the route of administration might be the cause of these diverse effects of inorganic tin compound. As mentioned earlier, monolayer culture of rabbit articular cartilage with tin compounds caused inhibition in the synthesis of core proteins, followed by a decrease in the synthesis of sulfated GAG.[33] In agreement with this result, our report also showed a decrease in the amount of sulfated GAG by culture of HAC with PGA(Sn). A study performed in our laboratory using HAC in a micromass culture system has already shown that PGA synthesized with organic tin catalyst caused a decrease in cell proliferation, but a significant increase in cell differentiation [29] and was completely contradictory to our present results. The molecular weight of PGA(Sn), and the type of tin product such as $SnCl_2$ and dibutyl tin were thought to be the key factor of different effects of chondrogenesis on HAC.

To the best of our knowledge, no other study has yet investigated the chondrogenic effects of PGA with inorganic tin as a catalyst, using HAC in a micromass culture system. This study is the first to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. Our observation revealed that low concentration of inorganic tin when used in the polymer

of PGA showed enhancing effects of tin compounds on chondrocytes in comparison to without tin polymer because of increase in the permeability of inorganic tin under the presence of PGA. However, further study is required for the application of this PGA(Sn) in clinical practice.

References

1. Yasui N, Osawa S, Ochi T, Nakashima H, Ono K. Primary culture of chondrocytes embedded in collagen gels. Exp Cell Biol 1982;50:92–100.
2. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;186:231–239.
3. Grandolfo M, D'Andrea P, Paoletti S, Martina M, Silvestrini G, Bonucci E, Vittur F. Culture and differentiation of chondrocytes entrapped in alginate gels. Calcif Tissue Int 1993;52:42–48.
4. Loty S, Sautier JM, Loty C, Boulekbache H, Kokubo T, Forest N. Cartilage formation by fetal rat chondrocytes cultured in alginate beads: a proposed model for investigating tissue-biomaterial interactions. J Biomed Mater Res 1998;42:213–222.
5. Perka C, Spitzer RS, Lindenhayn K, Sittinger M, Schultz O. Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. J Biomed Mater Res 2000;49:305–311.
6. Meinhart J, Fussenegger M, Hobling W. Stabilization of fibrin-chondrocyte constructs for cartilage reconstruction. Ann Plast Surg 1999;42:673–678.
7. Perka C, Schultz O, Lindenhayn K, Spitzer RS, Muschik M, Sittinger M, Burmester GR. Joint cartilage repair with transplantation of embryonic chondrocytes embedded in collagen-fibrin matrices. Clin Exp Rheumatol 2000;18:13–18.
8. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000;21:1145–1153.
9. George-Weinstein M, Gerhart JV, Foti GJ, Lash JW. Maturation of myogenic and chondrogenic cells in the preosmitic mesoderm of the chick embryo. Exp Cell Res 1994;211:263–274.
10. Ashammakhi N, Rokkanen P. Absorbable polyglycolide devices in trauma and bone surgery. Biomaterials 1997;18:3–9.
11. Middleton JC, Tipton JA. Synthetic biodegradable polymers as orthopedic devices. Biomaterials 2000;21:2335–2346.
12. Kohn J, Langer R. Bioresorbable and bioerodible materials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemon JE, editors. An Introduction to Materials in Medicine. San Diego: Academic Press; 1997. p 65–73.
13. Wong WH, Mooney DJ. Synthesis and properties of biodegradable polymers used as synthetic matrices for tissue engineering. In: Atala A, Mooney D, editors. Synthetic Biodegradable Polymer Scaffolds. Boston: Burkhauser; 1997. p 51–84.
14. Yaszemsky MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: Molecular, cellular and tissue strategies to engineer human bone. Biomaterials 1996;17:175–185.
15. Burg KJL, Porter S, Kellam JF. Biomaterials development for bone tissue engineering. Biomaterials 2000;21:2347–2359.
16. Tessa H, Sundback C, Hunter D, Cheney M, Vacanti JP. A polymer foam conduit seeded with Schwann cells promoted guided peripheral nerve regeneration. Tissue Eng 2000;6:119–127.
17. Bryan DJ, Holway AH, Wang KK, Silva AE, Trantolo DJ, Wise D, Summerhayes IC. Influence of glial growth factor and Schwann cells in a bioresorbable guidance channel on peripheral nerve regeneration. Tissue Eng 2000;6:129–138.
18. Evans GRD, Brandt K, Widmer MS, Lu L, Meszlenyi RK, Gupta PK, Mikos AG, Hodges J, Williams J, Gurlek A, Nabawi A, Lohman R, Patrick JCR. In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 1999;20:1109–1115.
19. Matsumoto K, Ohnishi K, Sekine T, Ueda H, Yamamoto Y, Kiyotani T, Nakamura T, Endo K, Shimizu Y. Use of a newly developed artificial nerve conduit to assist peripheral nerve regeneration across a long gap in dogs. ASAIO J 2000;46:415–420.
20. Salanki Y, D'eri Y, Platokhin A, Sh-Rozsa K. The neurotoxicity of environmental pollutants: the effect of tin (Sn2+) on acetylcholine-induced currents in growing pond snail neurons. Neurosci Behav Physiol 2000;30:63–73.
21. Gyori J, Platoshyn O, Carpenter DO, Salanki J. Effect of inorganic and organic tin compounds on Ach- and voltage-activated Na currents. Cell Mol Neurobiol 2000;20:591–604.
22. Chang LW. The neurotoxicology and pathology of organomercury, organolead, and organotin. J Toxicol Sci 1990;15:125–151.
23. de Mattos JC, Dantas FJS, Bezerra RJAC, Bernardo-Filho M, Cabral-Neto JB, Lage C, Leitao AC, Caldeira-de-Araujo A. Damage induced by stannous chloride in plasmid DNA. Toxicol Lett 2000;116:159–163.
24. Chao JS, Wei LY, Huang MC, Liang SC, Chen HH. Genotoxic effects of triphenyltin acetate and triphenyltin hydroxide on mammalian cells in vitro and in vivo. Mutat Res 1999;21:167–174.
25. De Santiago A, Aguilar-Santelises M. Ogranotin compounds decrease in vitro survival, proliferation and differentiation of normal human B lymphocytes. Hum Exp Toxicol 1999;18:619–624.
26. Yamaguchi M, Kitade M, Okada S. The oral administration of stannous chloride to rats. Toxicol Lett 1980;5:275–278.
27. Chang LW. Hippocampal lesions induced by trimethyltin in neonatal rat brain. Neurotoxicology 1984;5:205–215.
28. Tsuchiya T, Ikarashi Y, Arai T, Ohhashi J, Nakamura A. Improved sensitivity and decreased sample size in a cytotoxicity test for biomaterials: a modified colony microassay using a microplate and crystal violet staining. J Appl Biomater 1994;5:361–367.
29. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable polymers. Tissue Eng 2001;7:781–790.
30. Brown AN, Kim BS, Alsberg E, Mooney DJ. Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs. Tissue Eng 2000;6:297–305.
31. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987;237:1588–1595.
32. William SDF. Some observations on the role of cellular enzymes in the *in vivo* degradation of polymers. In: Syrett BC, Acharya A, editors. Corrosion and degradation of implant materials 1979. p 61–75.
33. Webber RJ, Dollins SC, Harris M, Hough AJ Jr. Effect of alkyltins on rabbit articular and growth-plate chondrocytes in monolayer culture. J Toxicol Environ Health 1985;16:229–242.
34. Yamaguchi M, Sugii K, Okada S. Inhibition of collagen synthesis in the femur of rats orally administered stannous chloride. J Pharm Dyn 1982;5:388–393.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Biomaterials 26 (2005) 5138–5144
Biomaterials
www.elsevier.com/locate/biomaterials

The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

Misao Nagahata[a,b,1], Ryusuke Nakaoka[a,1,*], Akira Teramoto[b], Koji Abe[b], Toshie Tsuchiya[a]

[a]*Division of Medical Devices, National Institute of Health Sciences, 1-81-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b]*Department of Functional Polymer Science, Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan*

Received 10 September 2004; accepted 7 January 2005

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.

*Corresponding author. Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 3 3700 9264; fax: +81 3 3707 6950.
E-mail addresses: nagahata@nihs.go.jp (M. Nagahata), nakaoka@nihs.go.jp (R. Nakaoka).
[1]These authors contributed equally to this work.

0142-9612/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2005.01.041

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].

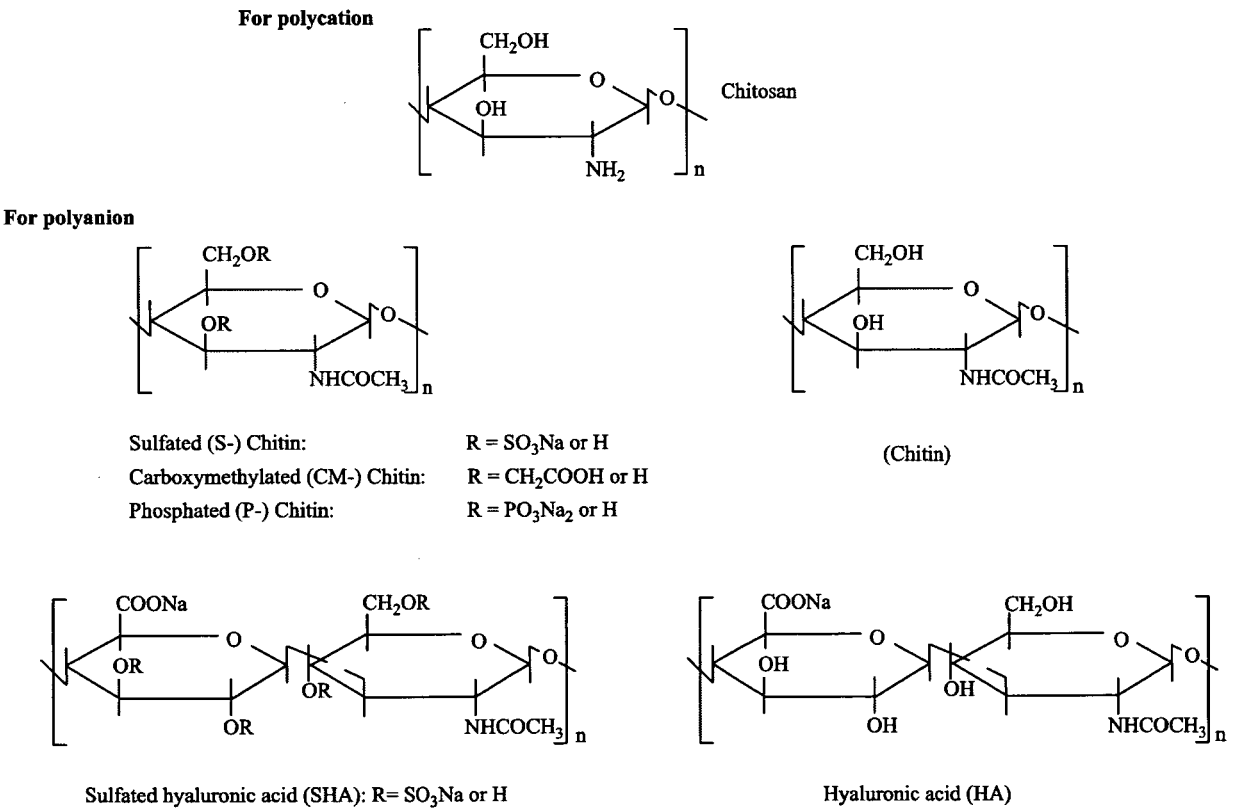

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

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.

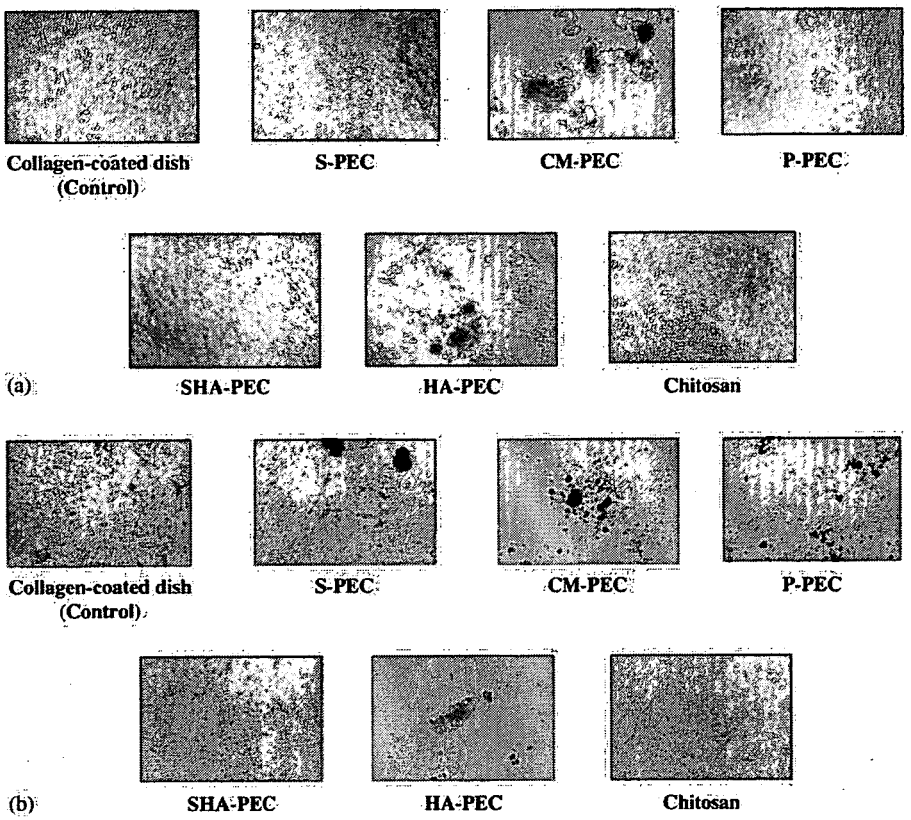

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

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 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

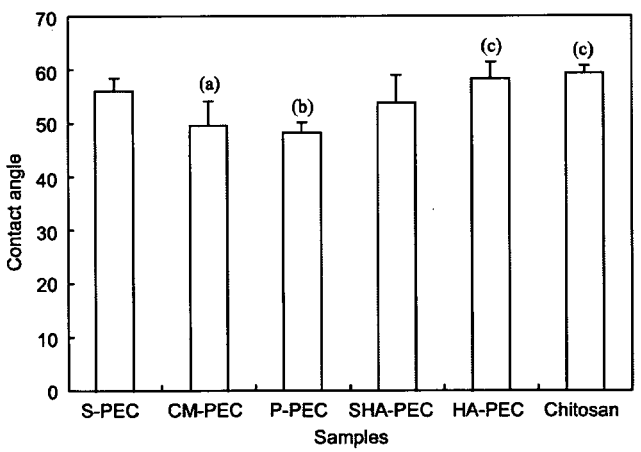

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

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.

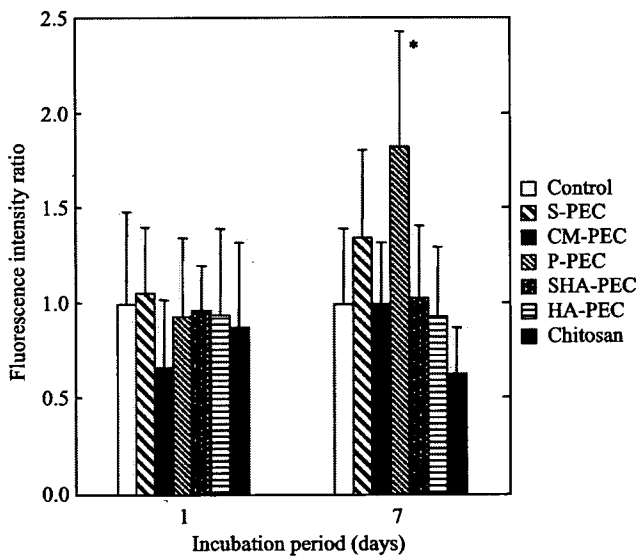

Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (*$p<0.01$ against control).

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.

Acknowledgements

This work was partially supported by Health and Labour Sciences Research Grants for Research on Advanced Medical Technology and Risk Analysis Research on Food and Pharmaceuticals by Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants for Research on Health Sciences focusing on Drug Innovation by the Japan Health Sciences Foundation, and Grant-in-Aid for 21st Century COE Program "Advanced Fiber Engineering" and Grant-in-Aid for Scientific Research (B), 2002 (14350495) by Ministry of Education, Science, Sports and Culture.

References

[1] Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development 1993;117:1183–98.

[2] Peterson WJ, Tachiki KH, Yamaguchi DT. Extracellular matrix alters the relationship between thymidine incorporation and proliferation of MC3T3-E1 cells during osteogenesis in vitro. Cell Prolif 2002;35:9–22.

[3] Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920–6.

[4] Mooney DJ, Mikos AG. Growing new organs. Sci Am 1999;280:38–43.

[5] Hori Y, Nakamura T, Matsumoto K, Kurokawa Y, Satomi S, Shimizu Y. Tissue engineering of the small intestine by acellular collagen sponge scaffold grafting. Int J Artif Organs 2001;24:50–4.

[6] Yamamoto M, Takahashi Y, Tabata Y. Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenetic protein. Biomaterials 2003;24:4375–83.

[7] Gamez E, Ikezaki K, Fukui M, Matsuda T. Photoconstructs of nerve guidance prosthesis using photoreactive gelatin as a scaffold. Cell Transplant 2003;12:481–90.

[8] Lavik E, Teng YD, Snyder E, Langer R. Seeding neural stem cells on scaffolds of PGA, PLA, and their co-polymers. Method Mol Biol 2002;198:89–97.

[9] Lieb E, Tessmar J, Hacker M, Fischbach C, Rose D, Blunk T, Mikos AG, Gopferich A, Schulz MB. Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers control adhesion and osteoblastic differentiation of marrow stromal cells. Tissue Eng 2003;9:71–84.

[10] Shin M, Ishii O, Sueda T, Vacanti JP. Contractile cardiac grafts using a novel nanofibrous mesh. Biomaterials 2004;25:3717–25.

[11] Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc Nat Acad Sci 2002;99:12025–30.

[12] Chang CH, Liu HC, Lin CC, Chou CH, Lin FH. Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. Biomaterials 2003;24:4853–8.

[13] Tsuchida E, Abe K. Interactions between macromolecules in solution and intermacromolecular complexes. Adv Polym Sci 1982;45:1–119.

[14] Hamano T, Teramoto A, Iizuka E, Abe K. Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblasts (HPLF) function. I. Three-dimensional structure of HPLF cultured PEC. J Biomed Mater Res 1998;41:257–69.

[15] Hamano T, Teramoto A, Iizuka E, Abe K. Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblasts (HPLF) function. II. Enhancement of HPLF differentiation and aggregation on PEC by *L*-ascorbic acid and dexamethasone. J Biomed Mater Res 1998;41:270–7.

[16] Hamano T, Chiba D, Nakatsuka K, Nagahata M, Teramoto A, Kondo Y, Hachimori A, Abe K. Evaluation of a polyelectrolyte complex (PEC) composed of chitin derivatives and chitosan, which promotes the rat calvarial osteoblast differentiation. Polym Adv Technol 2002;13:46–53.

[17] Maio AD, Vaga VL, Contreras JE. Gap junctions, homeostasis, and injury. J Cell Physiol 2002;191:269–82.

[18] Tsuchiya T, Hata H, Nakamura A. Studies on the tumor-promoting activity of biomaterials: inhibition of metabolic cooperation by polyetherurethane and silicone. J Biomed Mater Res 1995;29:113–9.

[19] Tsuchiya T, Takahara A, Cooper SL, Nakamura A. Studies on the tumor-promoting activity of polyurethanes: depletion of inhibitory action of metabolic cooperation on the surface of a polyalkyleneurethane but not a polyetherurethane. J Biomed Mater Res 1995;29:835–41.

[20] Nakaoka R, Tsuchiya T, Sakaguchi K, Nakamura A. Studies on in vitro evaluation for the biocompatibility of various biomaterials: inhibitory activity of various kinds of polymer microspheres on metabolic cooperation. J Biomed Mater Res 2001:279–84.

[21] Nakaoka R, Tsuchiya T, Nakamura A. The inhibitory mechanism of gap junctional intercellular communication induced by polyethylene and the restorative effects by surface modification with various proteins. J Biomed Mater Res 2001;57:567–74.

[22] Nagahata M, Tsuchiya T, Ishiguro T, Matsuda N, Nakatsuchi Y, Teramoto A, Hachimori A, Abe K. A novel function of *N*-cadherin and connexin 43: marked enhancement of alkaline phosphotase activity in rat calvarial osteoblast exposed to sulfated hyaluronan. Biochem Biophys Res Commun 2004;315:603–11.

[23] Tamada Y, Ikada Y. Fibroblast growth on polymer surfaces and biosynthesis of collagen. J Biomed Mater Res 1994;28:783–9.

[24] Ohyama M, Suzuki N, Yamaguchi Y, Maeno M, Otsuka K, Ito K. Effect of enamel matrix derivative on the differentiation of C2C12 cells. J Periodontol 2002;73:543–50.

[25] Wade MH, Trosko JE, Schlindler M. A fluorescence photobleaching assay of gap junctional-mediated communication between human cells. Science 1986;232:525–8.

[26] Kato S, Akagi T, Sugimura K, Kishida A, Akashi M. Evaluation of biological responses to polymeric biomaterials by RT-PCR analysis IV: study of c-myc, c-fos and p53 mRNA expression. Biomaterials 2000;21:521–7.

[27] Tanahashi M, Matsuda T. Surface functional group dependence on apatite formation on self-assembled monolayers in a simulated body fluid. J Biomed Mater Res 1997;34:305–15.

[28] Matsuda N, Horikawa M, Yoshida M, Watanabe M, Nagahata M, Teramoto A, Abe K. Enhanced DNA synthesis accompanied by constitutive phosphorylation of the ERK pathway in human fibroblasts cultured on a polyelectrolyte complex. Biomaterials 2003;24:4771–6.

Key Engineering Materials Vols. 288-289 (2005) pp. 409-412
online at http://www.scientific.net

Osteoblast Differentiation and Apatite Formation on Gamma-Irradiated PLLA Sheets

Kazuo Isama[a] and Toshie Tsuchiya[b]

Division of Medical Devices, National Institute of Health Sciences,

Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

[a]isama@nihs.go.jp, [b]tsuchiya@nihs.go.jp

Keywords: Poly(L-lactide), gamma-ray irradiation, osteoblast differentiation, apatite formation.

Abstract. The effects of the γ-irradiatted PLLA on the osteoblasts and apatite formation were investigated *in vitro*. The PLLA sheet was γ-ray irradiated at the dose of 10, 25 or 50 kGy. The mouse osteoblast-like MC3T3-E1 cells and normal human osteoblast NHOst cells were micromass cultured on the PLLA sheet for 2 weeks, and then the proliferation and differentiation of the cells were determined. The proliferations of MC3T3-E1 and NHOst cells hardly changed with increasing irradiation dose. However, the differentiations of MC3T3-E1 and NHOst cells increased with irradiation dose. On the other hand, the surface of the PLLA sheet after soaking in the medium without the cells was characterized by SEM, EDX, FT-IR and XPS. The hydroxyapatite was formed on the surface of the PLLA sheet after soaking, and the amount of hydroxyapatite increased with irradiation dose. In summary, the γ-irradiated PLLA increased the differentiation of osteoblasts and also increased apatite-forming ability even without the osteoblasts. The osteoblast differentiation was enhanced well in the apatite formation on the surface of PLLA after the γ-irradiation.

Introduction

Poly(L-lactide) (PLLA) has been well reported on a good osteocompatibility *in vivo* and *in vitro*. The γ-ray sterilized PLLA sample was implanted *in vivo*, and newly bone was formed around the PLLA implant [1]. It was not clear whether there was the effect of γ-irradiation on the formation of newly bone in this result. However, it was the fact that γ-irradiation decreased the molecular weight and mechanical strength of PLLA [2]. On the other hand, PLLA fibers formed bone-like apatite in a simulated body fluid [3]. It was reported that the apatite layer formed on the bioactive glass increased the attachment and initial proliferation of osteoblasts [4]. If the apatite-forming ability of PLLA is increased by γ-irradiation, there may be a good influence on osteoblasts cultured on the irradiated PLLA. Therefore, we clarified the effects of the γ-irradiatted PLLA sheet on the osteoblasts and apatite formation *in vitro*.

Materials and Methods

Materials. PLLA sheet with 0.3 mm thickness (Shimadzu Co., Japan) was γ-ray irradiated at the dose of 10, 25 or 50 kGy using ^{60}Co as the radiation source. The weight average molecular weight (Mw) of the unirradiated PLLA was 271,000 and the Mw's of the irradiated PLLA's at the dose