Hoechst dye efflux assay. Invest. Ophthalmol. Vis. Sci. 44, 2764–2773.
[33] Kumano, K. et al. (2001) Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. Blood 98, 3283–3289.
[34] Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D.T. (2002) Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 99, 2369–2378.
[35] Chojnacki, A., Shimazaki, T., Gregg, C., Weinmaster, G. and Weiss, S. (2003) Glycoprotein 130 signaling regulates Notch1 expression and activation in the self-renewal of mammalian forebrain neural stem cells. J. Neurosci. 23, 1730–1741.
[36] Sakakibara, S. et al. (1996) Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev. Biol. 176, 230–242.
[37] Belair, C.D., Yeager, T.R., Lopez, P.M. and Reznikoff, C.A. (1997) Telomerase activity: a biomarker of cell proliferation, not malignant transformation. Proc. Natl. Acad. Sci. USA 94, 13677–13682.
[38] Meeker, A.K. and Coffey, D.S. (1997) Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. A review. Biochemistry (Mosc). 62, 1323–1331.
[39] Cotsarelis, G., Sun, T.T. and Lavker, R.M. (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61, 1329–1337.
[40] Bickenbach, J.R. and Chism, E. (1998) Selection and extended growth of murine epidermal stem cells in culture. Exp. Cell. Res. 244, 184–195.
[41] Triel, C., Vestergaard, M.E., Bolund, L., Jensen, T.G. and Jensen, U.B. (2004) Side population cells in human and mouse epidermis lack stem cell characteristics. Exp. Cell. Res. 295, 79–90.
[42] Terunuma, A., Jackson, K.L., Kapoor, V., Telford, W.G. and Vogel, J.C. (2003) Side population keratinocytes resembling bone marrow side population stem cells are distinct from label-retaining keratinocyte stem cells. J. Invest. Dermatol. 121, 1095–1103.
[43] Li, A., Simmons, P.J. and Kaur, P. (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc. Natl. Acad. Sci. USA 95, 3902–3907.
[44] Tani, H., Morris, R.J. and Kaur, P. (2000) Enrichment for murine keratinocyte stem cells based on cell surface phenotype. Proc. Natl. Acad. Sci. USA 97, 10960–10965.
[45] Kim, D.S., Cho, H.J., Choi, H.R., Kwon, S.B. and Park, K.C. (2004) Isolation of human epidermal stem cells by adherence and the reconstruction of skin equivalents. Cell. Mol. Life Sci. 61, 2774–2781.

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Enhancement of Gap Junctional Intercellular Communication of Normal Human Dermal Fibroblasts Cultured on Polystyrene Dishes Grafted with Poly-*N*-isopropylacrylamide

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ABSTRACT

Technology developed to allow recovery of cells without enzyme treatment, involving a dish grafted with a thermoreactive polymer gel of poly-*N*-isopropylacrylamide (PIPAAm), was found to significantly enhance gap junctional intercellular communication (GJIC) in normal human dermal fibroblasts (NHDF cells). NHDF cells were cultured for 4 days on PIPAAm-grafted dishes irradiated with various doses of electron beams, and GJIC was assayed by the scrape-loading dye transfer method. The area of dye transfer was greater in the PIPAAm-grafted dishes than in the control culture dishes, indicating that the PIPAAm-grafted dishes enhanced the GJIC of NHDF cells. Connexin-43 (Cx43) expression was analyzed because Cx43 is considered to be a main component of the gap junctional channel. PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed significantly enhanced expression of Cx43-NP, Cx43-P1, and especially Cx43-P2. Enhanced expression of Cx43-P2, a functional transmembrane protein, may be related to the promotion of GJIC. These results suggest that the PIPAAm-grafted dish not only enables the enzyme-free recovery of a cell monolayer for use in the construction of a three-dimensional artificial tissue, but also significantly contributes to the enhancement of GJIC, which may partly promote tissue strength on the surface of the PIPAAm-grafted dish.

INTRODUCTION

GAP JUNCTIONS exist on the cell membrane and work as intercellular channels that allow the exchange of substances with molecular masses up to 1 kDa, such as ions, sugars, and amino acids, by the function called gap junctional intercellular communication (GJIC).[1–3] Gap junctions are constructed from transmembrane proteins, called connexins,[4,5] that form a hemichannel, called a connexon. GJIC is suggested to be well correlated with passage of metabolites,[6] cell proliferation,[7] and cell differentiation[8]; thus, enhancement of the function of the gap junction is supposed to be important in the differentiation of engineered tissue products, such as those involving heart cells.[9–11] Poly-*N*-isopropylacrylamide (PIPAAm)-grafted dishes, which were originally developed as a thermosensitive scaffold for cell culture, are useful to maintain the GJIC of tissues cultured on them because they do not require enzyme treatment, which destroys connexins.[12–14]

PIPAAm is a thermoresponsive polymer that has a low critical solution temperature of 32°C: hydrated PIPAAm

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has an extended chain conformation below 32°C and dehydrated PIPAAm has a collapsed chain conformation above 32°C.[15–27] This property of PIPAAm has been exploited in intelligent materials for drug delivery systems and chromatography technology.[16–23] The PIPAAm-grafted dish has been found to enable the recovery of cell monolayers easily without enzyme treatment because cells cannot adhere to a hydrophilic surface below 32°C.[24–26] Cell monolayers are the basic units used to construct three-dimensional tissues *in vitro*. Because a cell monolayer recovered without enzyme treatment maintains normal adhesive and junctional proteins, it can easily adhere to the other tissues or cell sheets to construct a three-dimensional artificial tissue.[27–29] Thus, the PIPAAm-grafted dish has the potential to enable the development of new techniques in tissue engineering.

Although the PIPAAm-grafted dish has made a new era in tissue engineering possible, its effects on connexin-43 (Cx43) expression and GJIC have not been studied well. These effects are important because Cx43 plays an important role in cell proliferation and cell differentiation.

In this study, GJIC and expression of Cx43 molecules were examined by scrape-loading dye transfer (SLDT) assay[30] and Western blotting, respectively, using NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams in order to clarify the safety and appropriateness of this material for the culture of artificial cultured tissues.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide monomer (NIPAAm) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Isopropyl alcohol was obtained from Dojindo (Kumamoto, Japan), and Lucifer yellow dye was from Molecular Probes (Eugene, OR).

Cell culture

Normal human dermal fibroblasts (NHDF cells; Sanko Junyaku, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (GIBCO DMEM; Invitrogen, San Diego, CA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and antibiotics (penicillin [100 units/mL]-streptomycin [100 units/mL]) (Invitrogen) at 37°C. NHDF cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of PIPAAm-grafted culture dishes

One hundred microliters of 40% NIPAAm dissolved in isopropyl alcohol was added to 35-mm dishes and irradiated with various doses of electron beams (25, 100, 250, or 500 kGy), using an area electron beam-processing system (Nissin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were then rinsed three times with ice-cold sterile water (2 ml) for 5 min, sealed, and dried under vacuum.

Cell morphology

NHDF cells were cultured on control and PIPAAm-grafted dishes. Confluent cells (after 4 days of culture) were fixed with formalin solution, stained with 3% Giemsa solution, and observed with an optical microscope.

Protein assay

The protein concentration of cells cultured on control and PIPAAm-grafted dishes was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Ten-microliter cell samples were

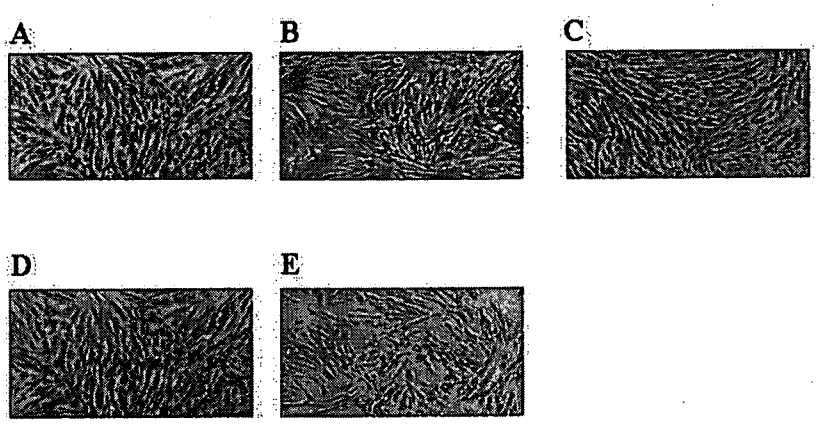

FIG. 1. Optical microscopy images of NHDF cells cultured on PIPAAm-grafted dishes. NHDF cells were cultured for 4 days on PIPAAm-grafted dishes prepared by irradiation with various doses of electron beams (0, 25, 100, 250, or 500 kGy). (**A**) Non-irradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

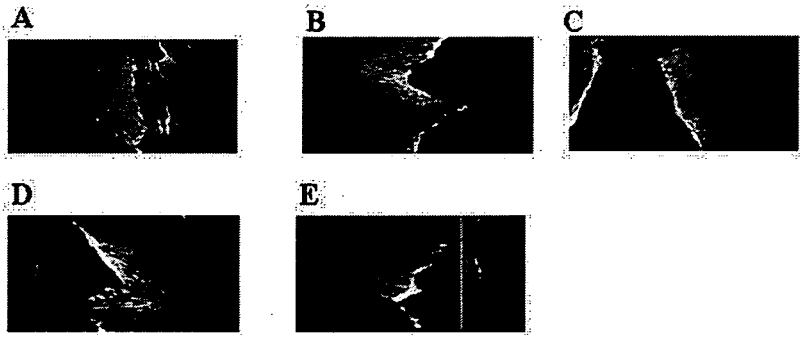

FIG. 2. Fluorescence of NHDF cells by SLDT assay. Transmission of Lucifer yellow into NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams was detected 5 min after scrape-loading. **(A)** Nonirradiated; **(B)** 25-kGy electron beam; **(C)** 100-kGy electron beam; **(D)** 250-kGy electron beam; **(E)** 500-kGy electron beam.

added to 200 μL of the working solution and incubated at 37°C for 30 min in a 96-well plate. Absorbance was then measured at 562 nm in accordance with the manufacturer's protocols.

Scrape-loading dye transfer assay

NHDF cells were seeded on control and PIPAAm-grafted dishes at a density of 1×10^5 cells/mL and cultured for 4 days to form a confluent monolayer. Confluent NHDF cells were washed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)], and the cell monolayer was scraped with a surgical blade. Fluorescent dye (Lucifer yellow; MW 457.2) at a concentration of 0.1% in PBS(+) was added.[30,31] Cells were exposed to the dye at 37°C for 5 min, and then the dye was discarded and the cells were washed four times with PBS(+). The distance that the dye had migrated was measured under a fluorescence microscope equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan). The dye migration was measured from the cut edge of the scrape to the edge of the dye front in the cells that were visually detectable.[30]

Western blotting

NHDF cells were cultured for 4 days. After being washed with ice-cold PBS(–) three times, the cells were lysed in 500 μL of lysis buffer (50 mM Tris-HCl [pH 6.8] containing 150 mM NaCl, 5 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) for 30 min on ice with shaking. The cell lysates were centrifuged (10,000 rpm) at 4°C for 20 min, and the supernatants were collected. The protein concentrations of the lysates were determined by BCA assay.

Equivalent amounts of protein sample were applied to 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and then transferred to a nitrocellulose membrane at 120 V for 60 min. The membrane was blocked with Block Ace (Yukijirusi, Tokyo, Japan) overnight at 4°C. After being washed for 30 min in PBS with 0.05% Tween 20, the membrane was incubated for 2 h with anti-Cx43 polyclonal antibody [diluted 1:1000 in PBS(–) with 0.05% Tween 20; Zymed Laboratories, South San Francisco, CA], followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000; Zymed Laboratories). The image was visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences/GE Healthcare, Little Chalfont, UK).

Statistical analysis

Significant differences between groups were evaluated by Student t test. Mean differences were considered significant when $p < 0.05$.

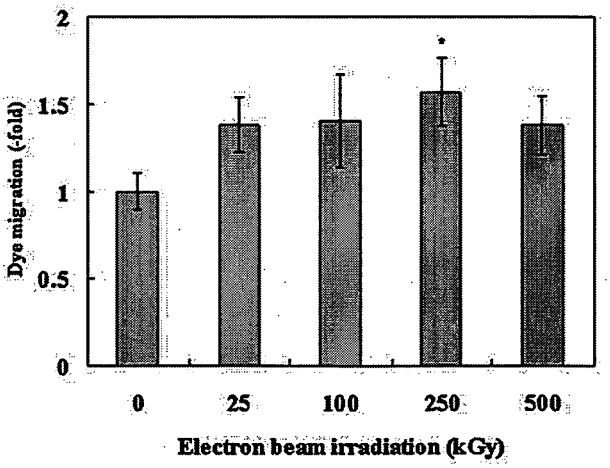

FIG. 3. Positive dye transfer in NHDF cells cultured on PIPAAm-grafted dishes. Transmission of Lucifer yellow was detected 5 min after scrape-loading in NHDF cells cultured on PIPAAm-grafted dishes irradiated with various electron beam doses (0, 25, 100, 250, or 500 kGy). Values represent means ± SD for three dishes. *Significant difference compared with control at $p < 0.05$ by t test.

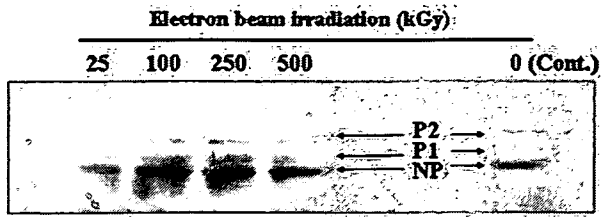

FIG. 4. Western blot of Cx43-NP, Cx43-P1, and Cx43-P2 expression; lysates of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy) were applied to SDS–polyacrylamide gels. Fractionated proteins in the gels were transferred to nitrocellulose membrane and immunoblotted with anti-Cx43 polyclonal antibody as described in Material and Methods. Images of Cx43 on Western blot were captured with an Image scanner and analyzed with NIH Image software.

RESULTS

The appearance of NHDF cells grown on PIPAAm-grafted dishes irradiated with various doses of electron beams are shown in Fig. 1. No significant differences were observed by optical microscopy analysis between cells grown in dishes irradiated with various doses of electron beams. These results suggest that PIPAAm-grafted dishes are not toxic to NHDF cells.

The SLDT assay showed that dye migration in cells cultured on PIPAAm-grafted dishes irradiated with electron beams (25, 100, or 500 kGy) was enhanced by about 1.4-fold compared with that on control dishes. Interestingly, the dye migration in cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was particularly enhanced, about 1.6 times higher than that on control dishes (Figs. 2 and 3). These results suggested that the GJIC of NHDF cells cultured on PIPAAm-grafted dishes was enhanced and that the GJIC on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was affected the most.

To further elucidate the effects of the PIPAAm grafting of culture dishes on GJIC, we analyzed the expression of Cx43, a transmembrane protein involved in GJIC. There are three forms of Cx43: Cx43-NP (nonphosphorylated Cx43), Cx43-P1 (monophosphorylated Cx43), and Cx43-P2 (another phosphorylated Cx43); Cx43-P2 is the most important and functional protein involved in GJIC. The results of Western blotting showed that the expression of Cx43-P1 and Cx43-P2 in NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, or 500 kGy of electron beams was considerably enhanced. Further, NHDF cells cultured on PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed enhanced Cx43-NP expression (Figs. 4 and 5A). The Cx43-P2 expression of cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam dose showed the highest value, about 46% higher than that of control dishes. Cells cultured on PIPAAm-grafted dishes irradiated with electron beam doses of 25, 100, and 500 kGy were shown to have enhanced total Cx43 expression. Cells cultured on PIPAAm-grafted dishes irradiated with 100- and 250-kGy electron beam doses showed the highest total Cx43 expression, about 36.6% higher than that of control dish (Fig. 5B).

The Cx43-P2 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, and 500 kGy correlated well with GJIC ($R^2 = 0.9398$).

DISCUSSION

Thermoresponsive PIPAAm-grafted dishes irradiated with electron beams have been used to culture cell monolayers because the monolayers can be recovered without enzyme treatment, making PIPAAm a useful material for tissue engineering.

It has been reported that junctional proteins, cellular adherence proteins on the cell membrane, interact via

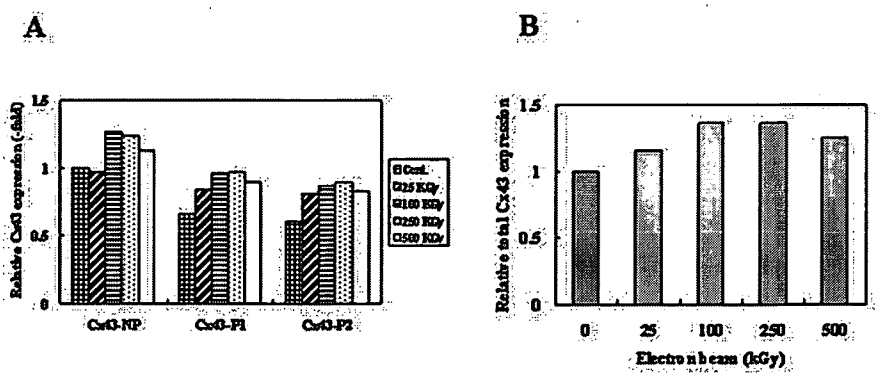

FIG. 5. Relative expression levels of Cx43-NP, Cx43-P1, and Cx43-P2 (**A**) and relative expression levels of total Cx43 (NP+P1+P2) (**B**) of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy).

GJIC.[31] In this study, an SLDT assay demonstrated that dye migration in cultured NHDF cells was significantly enhanced in all PIPAAm-grafted dishes tested. Therefore, the chemical structure of the PIPAAm surface may stimulate junctional proteins on the cell membrane, and the stimulated junctional proteins may induce the enhancement of GJIC.

Cx43 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with a 250-kGy electron beam changed significantly. Structural differences in PIPAAm triggered by the 250-kGy electron beam induced Cx43 protein expression by NHDF cells, probably by affecting the gene expression of NHDF cells. Further, total Cx43 expression was shown to be enhanced in cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (25, 100, 250, or 500 kGy). Differences due to the electron beam dose should be studied further.

Although the mechanism involved was not determined, it has been reported that basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF) enhance GJIC activity and the expression of Cx43.[32–35] If bFGF and KGF in FCS are adsorbed onto the PIPAAm surface, cells can efficiently access these growth factors from the PIPAAm surface, and GJIC may be enhanced. It is also reported that bFGF activates protein kinase A (PKA),[36] an important regulator of Cx43, promoting the phosphorylation of Cx43 and enhancing GJIC.[37] Therefore, bFGF adsorbed onto the PIPAAm surface may bind its receptor and induce the activation of PKA, resulting in an enhancement of GJIC on NHDF cells caused by the increase in Cx43-P2 band protein.

In the process of posttranslational change, Cx43-P2 becomes insoluble in Triton X-100.[38] Thus, not all Cx43-P2 may be included in the lysate, and some Cx43-P2 may have been included in the pellet. More Cx43-P2 may have existed than was detected in the present results obtained by Western blotting.

In this study, it was shown that the use of PIPAAm-grafted dishes irradiated with various doses of electron beams enhanced GJIC and Cx43 expression in cultured NHDF cells. This suggests that PIPAAm-grafted dishes may promote efficient tissue regeneration, because GJIC plays an important role in increasing tissue strength.[39]

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REFERENCES

1. Giaume, C., Tabernero, A., and Medina, J.M. Metabolic trafficking through astrocyte gap junctions. Glia **21,** 114, 1997.
2. Abdullah, K.M., Luthra, G., Bilski, J.J., Abdullah, S.A. Reynolds, L.P., Redmer, D.A., and Grazul-Bilska, A.T. Cell-to-cell communication and expression of gap junctional proteins in human diabetic and nondiabetic skin fibroblasts. Endocrine **10,** 35, 1999.
3. Grossman, H.B., Liebert, M., Lee, I.W., and Lee, S.W. Decreased connexin expression and intercellular communication in human bladder cancer cells. Cancer Res. **54,** 3062, 1994.
4. Vera, B., Sanchez-Abarca, L.I., Bolanos, J.P., and Medina, J.M. Inhibition of astrocyte gap junctional communication by ATP depletion is reversed by calcium sequestration. FEBS Lett. **392,** 225, 1996.
5. Bukauskas, F.F., Jordan, K., Bukauskiene, A., Bennett, M.V., Lampe, P.D., Laird, D.W., and Verselis, V.K. Clustering of connexin 43–enhanced green fluorescent protein gap junction channels and functional coupling in living cells. Proc. Natl. Acad. Sci. U.S.A. **97,** 2556, 2000.
6. Giaume, C., Tabernero, A., and Medina, J.M. Metabolic trafficking through astrocytic gap junctions. Glia **21,** 114, 1997.
7. Tabernero, A., Jimenez, C., Velasco, A., Giaume, C., and Medina, J.M. The enhancement of glucose uptake caused by the collapse of gap junction communication is due to an increase in astrocyte proliferation. J. Neurochem. **78,** 890, 2001.
8. Tsuchiya, T. A useful marker for evaluating tissue-engineered products: Gap-junctional communication for assessment of the tumor-promoting action and disruption of cell differentiation in tissue-engineered products. J. Biomater. Sci. Polym. Ed. **11,** 947, 2000.
9. Giaume, C., Marin, P., Cordier, J., Glowinski, J., and Premont, J. Adrenergic regulation of intercellular communications between cultured striatal astrocytes from the mouse. Proc. Natl. Acad. Sci. U.S.A. **88,** 5577, 1991.
10. Warn-Cramer, B.L., Cottrell, G.T., Burt, J.M., and Lau, A.F. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinases. J. Biol. Chem. **273,** 9188, 1998.
11. Shimizu, T., Yamato, M., Isoi, Y., Akutsu, T., Setomaru, T., Abe, K., Kikuchi, A., Umezu, M., and Okano, T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ. Res. **22,** e40, 2002.
12. Nandkumar, M.A., Yamato, M., Kushida, A., Konno, C., Hirose, M., Kikuchi, A., and Okano, T. Two-dimensional cell sheet manipulation of heterotypically co-cultured lung cells utilizing temperature-responsive culture dishes results in long-term maintenance of differentiated epithelial cell functions. Biomaterials **23,** 1121, 2002.
13. Kushida, A., Yamato, M., Kikuchi, A., and Okano, T. Two-dimensional manipulation of differentiated Madin–Darby canine kidney (MDCK) cell sheets: The noninvasive harvest from temperature-responsive culture dishes and transfer to other surfaces. J. Biomed. Mater. Res. **54,** 37, 2001.
14. Shimizu, T., Yamato, M., Akutsu, T., Shibata, T., Isoi, Y., Kikuchi, A., Umezu, M., and Okano, T. Electrically com-

municating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. J. Biomed. Mater. Res. **60,** 110, 2002.
15. Takezawa, T., Mori, Y., and Yoshizato, K. Cell culture on a thermo-responsive polymer surface. Biotechnology **8,** 854, 1990.
16. Chung, J.E., Yokoyama, M., Yamato, M., Aoyagi, T., Sakurai, Y., and Okano, T. Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(*N*-isopropylacrylamide) and poly (butylmethacrylate). J. Control. Release **62,** 115, 1999.
17. Chung, J.E., Yokoyama, M., and Okano, T. Inner core segment design for drug delivery control of thermo-responsive polymeric micelles. J. Control. Release **65,** 93, 2000.
18. Kurisawa, M., Yokoyama, M., and Okano, T. Gene expression control by temperature with thermo-responsive polymeric gene carriers. J. Control. Release **69,** 127, 2000.
19. Kobayashi, J., Kikuchi, A., Sakai, K., and Okano, T. Aqueous chromatography utilizing pH-/temperature-responsive polymer stationary phases to separate ionic bioactive compounds. Anal. Chem. **73,** 2027, 2001.
20. Kikuchi, A., and Okano, T. Pulsatile drug release control using hydrogels. Adv. Drug Deliv. Rev. **54,** 53, 2002.
21. Kanazawa, H., Sunamoto, T., Ayano, E., Matsushima, Y., Kikuchi, A., and Okano, T. Temperature-responsive chromatography using poly (*N*-isopropylacrylamide) hydrogel-modified silica. Anal. Sci. **18,** 45, 2002.
22. Kobayashi, J., Kikuchi, A., Sakai, K., and Okano, T. Aqueous chromatography utilizing hydrophobicity-modified anionic temperature-responsive hydrogel for stationary phases. J. Chromatogr. A **958,** 109, 2002.
23. Yoshizako, K., Akiyama, Y., Yamanaka, H., Shinohara, Y., Hasegawa, Y., Carredano, E., Kikuchi, A., and Okano, T. Regulation of protein binding toward a ligand on chromatographic matrixes by masking and forced-releasing effects using thermoresponsive polymer. Anal. Chem. **74,** 4160, 2002.
24. Kikuchi, A., Okuhara, M., Karikusa, F., Sakurai, Y., and Okano, T. Two-dimensional manipulation of confluently cultured vascular endothelial cells using temperature-responsive poly(*N*-isopropylacrylamide)-grafted surfaces. J. Biomater. Sci. Polym. Ed. **9,** 1331, 1998.
25. Kushida, A, Yamato, M., Konno, C., Kikuchi, A., Sakurai, Y., and Okano, T. Temperature-responsive culture dishes allow nonenzymatic harvest of differentiated Madin–Darby canine kidney (MDCK) cell sheets. J. Biomed. Mater. Res. **51,** 216, 2000.
26. Shimizu, T., Yamato, M., Kikuchi, A., and Okano, T. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. Tissue Eng. **7,** 141, 2001.
27. Hirose, M., Yamato, M., Kwon, O.H., Harimoto, M., Kushida, A., Shimizu, T., Kikuchi, A., and Okano, T. Temperature-responsive surface for novel co-culture systems of hepatocytes with endothelial cells: 2-D patterned and double layered co-cultures. Yonsei Med. J. **41,** 803, 2000.
28. Yamato, M., Utsumi, M., Kushida, A, Konno, C., Kikuchi, A., and Okano, T. Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng. **7,** 473, 2001.
29. Harimoto, M., Yamato, M., Hirose, M., Takahashi, C., Isoi, Y., Kikuchi, A., and Okano, T. Novel approach for achieving double-layered cell sheets co-culture: Overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J. Biomed. Mater. Res. **62,** 464, 2002.
30. el-Fouly, M.H, Trosko, J.E., and Chang, C.C. Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication. Exp. Cell Res. **168,** 422, 1987.
31. Defamie, N., Mograbi, B., Roger, C., Cronier, L., Malassine, A., Brucker-Davis, F., Fenichel, P., Segretain, D., and Pointis, G. Disruption of gap junctional intercellular communication by lindane is associated with aberrant localization of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells. Carcinogenesis **22,** 1537, 2001.
32. Pepper, M.S., and Meda, P. Basic fibroblast growth factor increases junctional communication and connexin 43 expression in microvascular endothelial cells. J. Cell. Physiol. **153,** 196, 1992.
33. Nadarajah, B., Makarenkova, H., Becker, D.L., Evans, W.H., and Parnavelas, J.G. Basic FGF increases communication between cells of the developing neocortex. J. Neurosci. **18,** 7881, 1998.
34. Park, J.U., and Tsuchiya, T. Increase in gap junctional intercellular communication by high molecular weight hyaluronic acid associated with fibroblast growth factor 2 and keratinocyte growth factor production in normal human dermal fibroblasts. Tissue Eng. **8,** 419, 2002.
35. Doble, B.W., and Kardami, E. Basic fibroblast growth factor stimulates connexin-43 expression and intercellular communication of cardiac fibroblast. Mol. Cell. Biochem. **143,** 81, 1995.
36. Pursiheimo, J.P., Jalkanen, M., Tasken, K., and Jaakkola, P. Involvement of protein kinase A in fibroblast growth factor-2-activated transcription. Proc. Natl. Acad. Sci. U.S.A. **97,** 168, 2000.
37. Faucheux, N., Zahm, J.M., Bonnet, N., Legeay, G., and Nagel, M.D. Gap junction communication between cells aggregated on a cellulose-coated polystyrene: Influence of connexin 43 phosphorylation. Biomaterials **25,** 2501, 2004.
38. Musil, L.C., and Goodenough, D.A. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J. Cell Biol. **115,** 1357, 1991.
39. Gutstein, D.E., Morley, G.E., Tamaddon, H., Vaidya, D., Schneider, M.D., Chen, J., Chien, K.R., Stuhlman, H., and Fishman, G.I. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circ. Res. **88,** 333, 2001.

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ORIGINAL ARTICLE

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Biodegradable polymers in chondrogenesis of human articular chondrocytes

Abstract The aim of this study was to evaluate the potential role of polyglycolic acid (PGA), poly(glycolic acid-ε-caprolactone) (PGCL), poly(L-lactic acid-glycolic acid) (PLGA), poly(L-lactic acid-ε-caprolactone, 75:25 (w/w)) [P(LA-CL)25], poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)], and fullerene C-60 dimalonic acid (DMA) in cartilage transplants. After 4 weeks of culture of human articular cartilage, the levels of cell proliferation and differentiation and the expression of cartilage-specific matrix genes were estimated. The relationship between cell differentiation and gap junction protein connexin 43 (Cx43) was also evaluated. All materials except PCL(Ti) retained cell proliferation activities similar to the controls. Cell differentiation levels from the highest to the lowest were in the following order: PGA >> PLGA > PGCL > Control = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C-60 DMA. Expression of the collagen type II gene was selectively upregulated for PGA, PGCL, and PLGA and slightly increased for P(LA-CL)25 polymers but was downregulated for fullerene C-60 DMA. Aggrecan gene expression was strongest with PGA and was consistently expressed with other matrices, especially with PGCL and PLGA. However, the expression patterns of the connexin 43 gene were different from the former two genes. Multiple regression analysis revealed a high correlation between cartilage proteoglycans production and expression levels of these three genes.

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Key words Human articular chondrocytes · Biodegradable polymers · Matrix gene · Connexin 43

Introduction

A shortage of donor tissue restricts the successful application of tissue reconstruction for various cartilage injuries. Tissue engineering is a relatively new and promising field directed at the evolution of new tissues that will offer hope to orthopedic patients with a variety of injuries. To permit repair of cartilage defects, many researchers are turning toward a tissue engineering approach involving cultured cells and biomaterials. Although these biomaterials, especially polyglycolic acid (PGA) and poly(L-lactic acid) (PLLA), play an increasingly important role in orthopedics, adverse reactions to these biomaterials have been reported in animal experiments. PLLA produces toxic substances due to acidic degradation,[1] and long-term implants of PLLA produced tumorigenicity in rats.[2] Despite these setbacks, numerous studies have documented the biocompatibility of these bioabsorbable polymers.[3–7] PLLA, PGA, and their copolymers also have been used in clinical practice.[5,8] More recent studies have indicated that copolymers of glycolic acid promoted peripheral nerve regeneration in a rat model.[9,10] These polymers are degraded by hydrolysis and enzymatic activity and have a range of mechanical and physical properties that can be engineered appropriately to suit a particular application.

Knowledge of the biological interactions between chondrocytes and biodegradable polymers is needed to design novel biomaterials and to develop new strategies for cartilage repair. Therefore, further experimental elucidation of these polymers, their combination with other biomaterials, and new materials to find good substrates is essential to attain satisfactory conditions for their clinical application. In this study, along with PGA and poly(L-lactic acid-glycolic acid) (PLGA), we investigated the copolymer poly(glycolic acid-ε-caprolactone) (PGCL), the copolymer poly(L-lactic acid-ε-caprolactone) 75:25 (w/w) P(LA-

CL)25, and poly-ε-caprolactone (tetrabutoxy titanium) [PCL(Ti)] to determine their effects on human articular chondrocyte (HAC) proliferation, differentiation, and phenotypic expression with the aim of clarifying their suitability as carriers for future clinical cartilage transplants. Fullerene C-60 dimalonic acid (DMA) has been reported to stimulate[11] and inhibit[12] proliferation and differentiation of rat embryonic limb bud cells and mouse embryo midbrain cells, respectively, and in the present study we also investigated the effect of fullerene C-60 DMA on HACs.

Gap junctions are intercellular channels supporting direct cell-to-cell communication and tissue integration.[13] Connexins, the family of proteins that form vertebrate gap junctions, play key roles during development and in the adult. Among the 19 connexins that have been identified in mammals, the gap junction protein connexin 43 (Cx43) is the most abundant member of the channel-forming proteins in chondrocytes.[14,15] The distribution of Cx43 in hyaline cartilage and in the perichondrium of mouse and rat knee joints suggested a possible involvement of connexins in cartilage development.[16] It has been indicated that the early stage of in vitro chondrocyte differentiation is the formation of cell condensations and the ability to establish cell-to-cell communication. Cx43, together with other molecular mechanisms, mediates the condensation phase of chondrogenesis.[17] In the present study, we investigated the role of gap junctional protein Cx43 in the process of chondrocyte differentiation.

Materials and methods

Materials

HACs from knee joints and chondrocyte growth medium were commercially obtained from BioWhittaker (Walkersville, MD, USA). Chondrocyte growth medium contains bovine insulin, basic fibroblast growth factor, insulin-like growth factor-1, transferrin, gentamicin sulfate, and fetal bovine serum (5% v/v). PGA (mw 3000) and PLGA (mw 5000) were purchased from Nakalai Tesque (Kyoto, Japan) and PGCL (mw 3000) was from Taki Chemical (Hyogo, Japan). P(LA-CL)25 (mw 10000) and PCL(Ti) (mw 130000) were synthesized in our laboratory and fullerene C-60 DMA was obtained from Dr. T. Mashino.[18]

Synthesis of P(LA-CL)25

L-Lactide (Tokyo Kasei Kogyo, Tokyo, Japan) 7.5 g and caprolactone (Wako Pure Chemical Industries, Osaka, Japan) 2.5 g were put into a reactor as monomers. As a catalyst, tetrabutoxy titanium (Wako) 0.03 g was added. Furthermore, *n*-octyl alcohol (Wako) 0.001 g was added. These were completely dissolved in methylene chloride (Wako) 50 mL at room temperature. Methylene chloride was removed by decompression and a uniform mixture was left. The reactor was filled with nitrogen and was sealed. The contents were mixed and heated to 140°C. Polymerization was carried out for 4 h. After the reaction, the reactant was cooled to room temperature, and was dissolved in tetrahydrofuran 100 mL. The solution was dropped into cold methanol and a colorless precipitate was obtained. This was dried under reduced pressure and precipitation was done once again. This was again dried under reduced pressure and the polymer was obtained. The yield was 58.2% (5.82 g).

Synthesis of PCL(Ti)

Synthesis was done using the same method as described for the synthesis of P(LA-CL)25 except that the monomer was only caprolactone (Wako). The yield was 87.1% (8.71 g).

Preparation of materials

PGA, PGCL, PLGA, and P(LA-CL)25 were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 50 μg/0.8 μl of DMSO (Sigma-Aldrich, Irvine, CA, USA) and then dissolved in chondrocyte growth medium to give a final concentration of 50 μg/ml. PCL(Ti) was dissolved in tetrahydrofran (THF) at a concentration of 5 mg of PCL/ml of THF. Glass wells were coated with this solution to give a final concentration of 2 mg PCL(Ti)/well. A homogenous solution of fullerene C-60 DMA was made with the chondrocyte growth medium.

Cell culture

In vitro high-density micromass cultures of HACs were initiated by spotting 4×10^5 cells in 20 μl of medium onto each well of 12-well microplates for tissue culture (Costar Type 3513, Corning, Corning, NY, USA) and PCL(Ti)-coated glass wells (diameter 22 mm). After 2 h in a 5% CO_2 incubator at 37°C, the wells were flooded with chondrocyte growth medium (2 ml/well). The medium was supplemented with DMSO (0.8 μl/ml), PGA (50 μg/ml), PGCL (50 μg/ml), PLGA (50 μg/ml), P(LA-CL)25 (50 μg/ml), or fullerene C60 DMA (50 μg/ml). HACs cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and culture was continued for 4 weeks.

Cell morphology assay

Cell morphology was determined by inverted light microscopy. Twice weekly observations were done and photographs were taken with Fuji film.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource International, Camarillo, CA, USA) assay after 4 weeks of culture, as previously described.[18] The assay

demonstrates the metabolic activity of the cells by detection of mitochondrial activity. The indicator dye alamar blue is incorporated into the cells and reduced and excreted as a fluorescent product. At the end of the 4-week culture period, the medium from all wells was discarded and the culture wells and three blank wells were filled with 1 ml/well of 5% alamar blue solution in fresh medium. The culture plates were incubated at 37°C for 4h. After the incubation period, two aliquots of 100µl of solution from each well were transferred to new wells of a Costar 96-well tissue culture microplate (Costar Type 3595, Corning). The extent of cell proliferation was quantitated by a Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535nm for excitation and 590nm for emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from experimental values to eliminate background readings.

Proteoglycan production assay

Proteoglycans are typical components of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage-specific proteoglycans with alcian blue (Wako) as described previously.[11,19] Briefly, the cultures and three blank wells were stained overnight at 4°C (0.5ml/well) with 1% (v/v) alcian blue, pH 1.0. The alcian blue solution was then removed and the micromass cultures and blank wells were rinsed with 3% (v/v) acetic acid and distilled water to completely remove the free dye. The cartilage proteoglycans were extracted using 4-M guanidine hydrochloride, and the absorbance was measured at a wavelength of 600nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, Winooski, VT, USA). Blank values were subtracted from experimental values to eliminate background readings.

RNA harvest

After the 4-week culture period, RNA was extracted from all matrices except the PCL(Ti) matrix. For the PCL(Ti) matrix, we did not have enough samples to harvest RNA because cells from 50% of the cultured wells became detached overnight following cell spotting. Total cellular RNA was extracted from cultured cells of four wells (for each material) in 0.5ml Trizol reagent (Life Technologies, Frederick, MD, USA) according to the manufacturer's instructions. The concentration of total RNA was determined using a UV spectrophotometer (Gene Quanta, Pharmacy Biotech, Piscataway NJ, USA) at 260nm.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study were collagen type II and aggrecan. The gap junction protein gene Cx43 was also studied. Single-strand cDNA was prepared from 1µg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After optimization of PCR conditions, subsequent PCR was performed with 4µg of cDNA in a 20-µl reaction mixture (10 × PCR buffer 2µl, dNTP 1.6µl, forward and reverse primer 0.4µl, Taq DNA polymerase 0.1µl, and distilled water to make up 20µl). The codon sequence used for the primer sets was as follows:

Collagen type II:
forward 5′-GGCAATAGCAGGTTCACGTACA-3′
reverse 5′-CGATAACAGTCTTGCCCCACTT-3′

Aggrecan:
forward 5′-TCGAGGACAGCGAGGCC-3′
reverse 5′-TCGAGGGTGTAGCGTGTAGAGA-3′.
Connexin 43 (*Homo sapiens*):
forward 5′-ATGGGTGACTGGAGCGCCTTAGGCAAACTC-3′
reverse 5′-GACCTCGGCCTGATGACCTGGAGATCTAG-3′

For collagen type II and Cx43, an initial denaturation step at 94°C was carried out for 5min, followed by 40 cycles of 94°C for 1min, 60°C for 1min, and 72°C for 1min, with a final extension at 72°C for 10min. For aggrecan, an initial denaturation at 95°C was carried out for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 1min, and 72°C for 1min), with a final extension at 72°C for 5min. The polymerization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was accomplished by 25 cycles with a corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for visualization of collagen type II and aggrecan and on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, GAPDH:

forward 5′-CCCATCACCATCTTCCAGGAGCGAGA-3′
reverse 5′-TGGCCAAGGTCATCCATGACAACTTTGG-3′.

Statistical analysis

Comparing the control with samples exposed to various materials assessed the statistical significance of the cell proliferation and cartilage proteoglycans production. Student's *t* test was used to assess the statistical significance. Statistical significance was taken as $P < 0.05$. Data were indicated as the mean ± SD (standard deviation). Four or five cultures were run for each biomaterial. All experiments were repeated at least twice, and similar results were obtained.

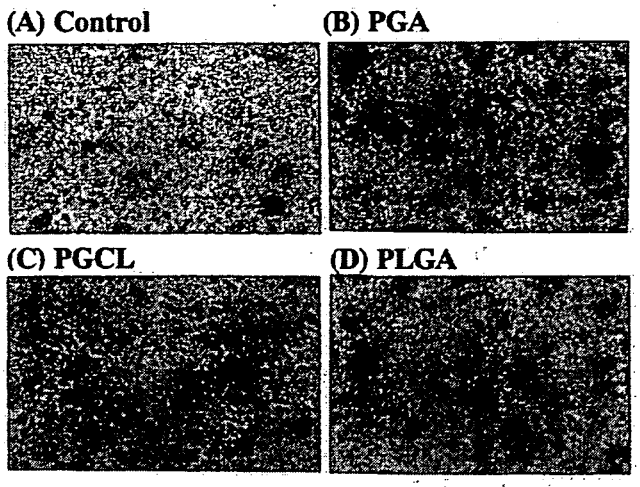

Fig. 1A–D. Light microscopic appearance of cultured human articular chondrocytes spotted as a high-density micromass culture with different biodegradable polymers for 4 weeks. **A** Control, **B** polyglycolic acid (PGA), **C** poly(glycolic acid-ε-caprolactone) (PGCL), **D** poly(L-lactic acid-glycolic acid) (PLGA). Original magnification ×200

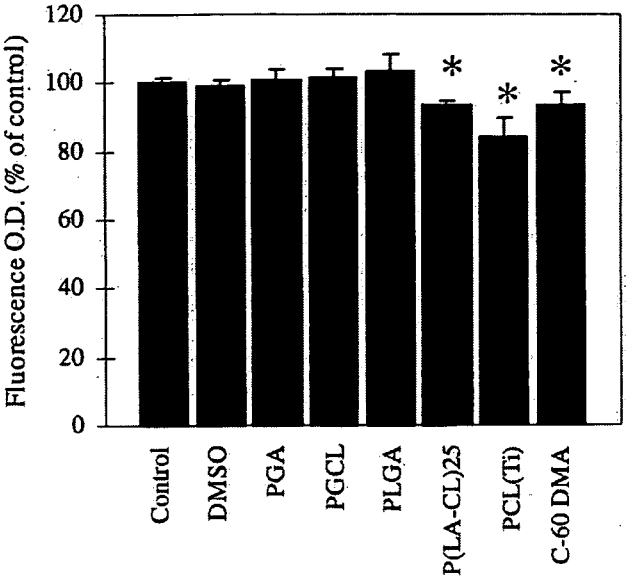

Fig. 2. Cell proliferation of human articular chondrocytes as determined by alamar blue assay after culturing with synthetic biodegradable polymers for 4 weeks. The proliferation in all samples exposed to dimethyl sulfoxide (*DMSO*) and biomaterials were calculated as a percentage of control values. *P(LA-CL)25*, poly(L-lactic acid-ε-caprolactone) 75:25 (w/w); *PCL(Ti)*, poly-ε-caprolactone (tetrabutoxy titanium); *C-60 DMA*, fullerene C-60 dimalonic acid. $*P < 0.05$ and *error bars* represent standard deviations of the mean

Results

Cell morphology

Cells were aggregated as high-density micromass cultures 2 h after cell spotting. After 4 weeks of culture, the chondrocytes mainly formed a uniform sheet of chondrogenic cells with nodules. The cartilage nodules were first observed in the first week of the culture. These nodules were better visualized by staining the proteoglycans with alcian blue after 4 weeks of culture. The control cells showed less nodule formation and they were poorly defined (Fig. 1A). The cultures exposed to the PGA and PLGA had more distinct nodules and greater numbers of nodule formations than the controls (Figs. 1B and 1D). The nodules formed in the culture exposed to PGCL were less distinct and fewer in number than the nodules in the cultures exposed to PGA and PLGA, but were more distinct and numerous than the nodules of the control cultures (Fig. 1C). After alcian blue staining, light microscopic examination also revealed that PGA-, PGCL-, and PLGA-treated cultures contained denser extracellular matrix (ECM) than the controls. Cells extended from the edge of all micromass cultures, and the extending cells were spindle-shaped.

Cell proliferation assay

The proliferation rates of all the matrices are shown in Fig. 2, with error bars representing the standard deviation of the mean. All values for the samples exposed to the biomaterials were expressed as a percentage of the control average value, which was taken as 100%. The effect of DMSO on cell proliferation was not significant (99.3% ± 1.6%). The cell proliferations for PGA, PGCL, and PLGA were fairly parallel to that of control cell proliferation. The cell proliferation for P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were significantly inhibited compared to the control. The inhibitions for P(LA-CL)25 and fullerene C-60 DMA were mainly due to the small variation of the standard deviation. Despite being significantly different from the control, both proliferation values were fairly close to the control proliferation value.

Therefore, from the standpoint of cell proliferation, all materials except for PCL(Ti) remained viable candidates for tissue engineering. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti), and fullerene C-60 DMA were 101% ± 2.7%, 101.6% ± 2.2%, 103.5% ± 4.8%, 93.2% ± 1.4%, 84.3% ± 5.1%, and 93.6% ± 3.7%, respectively.

Proteoglycan synthesis

The proteoglycans bound with alcian blue were extracted with 4-M guanidine hydrochloride. Their levels were expressed as a percentage of the average control value, which was taken as 100% (Fig. 3). The intensity of alcian blue staining was found to be higher in PGA-, PGCL-, and PLGA-containing cultures than in the control culture. Among the biomaterials, PGA caused a significant 3.1-fold increase in cartilage proteoglycans compared to the control ($P < 0.05$). The samples exposed to PGCL (116.2% ± 10.1%) and PLGA (128.4% ± 11.1%) also produced

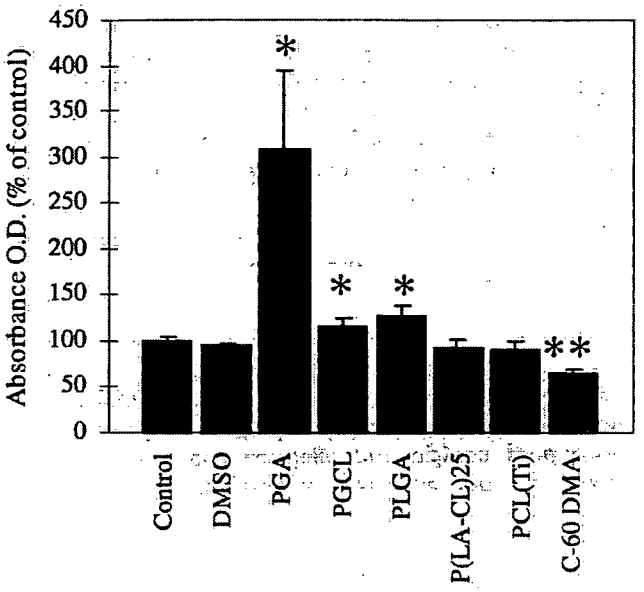

Fig. 3. Cartilage proteoglycan content of human articular chondrocytes as determined by the alcian blue staining method after culturing with synthetic biodegradable polymers for 4 weeks. The values are expressed as a percentage of control values. $^{*}P < 0.05$ and $^{**}P < 0.01$

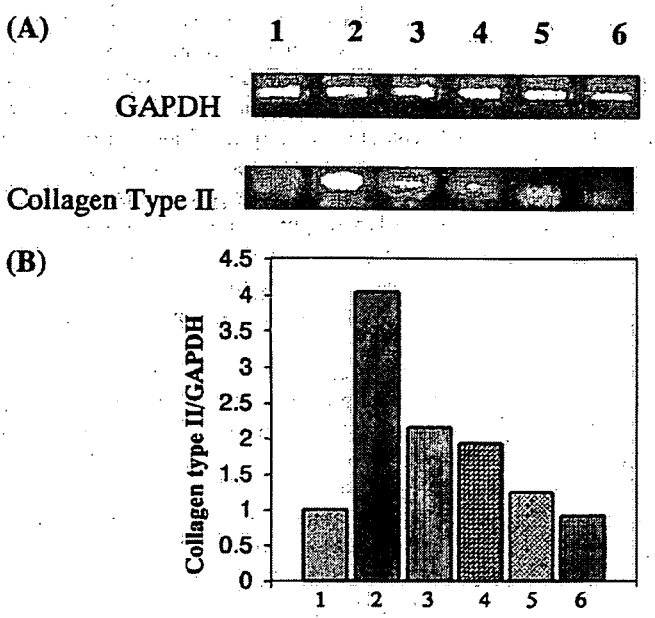

Fig. 4. Reverse transcription polymerase chain reaction (RT-PCR) analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of collagen type-II gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for comparing the level of expression. **A** Lane 1, Control; lane 2, PGA; lane 3, PGCL; lane 4, PLGA; lane 5, P(LA-CL)25; lane 6, Fullerene C-60 DMA. **B** Bar 1, Control; bar 2, PGA; bar 3, PGCL; bar 4, PLGA; bar 5, P(LA-CL)25; bar 6, Fullerene C-60 DMA

significantly higher cartilage proteoglycans than the control. Copolymers P(LA-CL)25 (92.7% ± 10.5%) and PCL(Ti) (90.8% ± 9.1%) did not induce significant changes in cartilage proteoglycans compared to the control. Fullerene C60 DMA acted as a potent inhibitor (66.1% ± 4.7%) and caused a significant inhibition of cartilage proteoglycans ($P < 0.01$) compared to the control. The effect of DMSO (96% ± 1.1%) on cell differentiation was negligible.

Extracellular matrix gene expression

RT-PCR and corresponding National Institutes of Health (NIH) image analysis showed that all matrices consistently supported the expression of the collagen type II gene and that the PGA matrix had the strongest induction (Fig. 4). Slight increases in expression of the collagen type II gene were noted with PGCL, PLGA, and P(LA-CL)25 matrices. Expression of the collagen type II gene for fullerene C60 DMA was similar to the control. The PGA matrix also showed the strongest induction of the aggrecan gene (Fig. 5). Aggrecan gene expression was slightly increased in PGCL and PLGA matrices. The P(LA-CL)25 matrix caused an expression of this gene similar to that of the control, but the fullerene C60 DMA matrix caused decreased expression of this gene.

Expression of gap junction protein connexin 43 gene

To determine the expression of gap junctions during in vitro chondrocyte differentiation, RT-PCR and corresponding

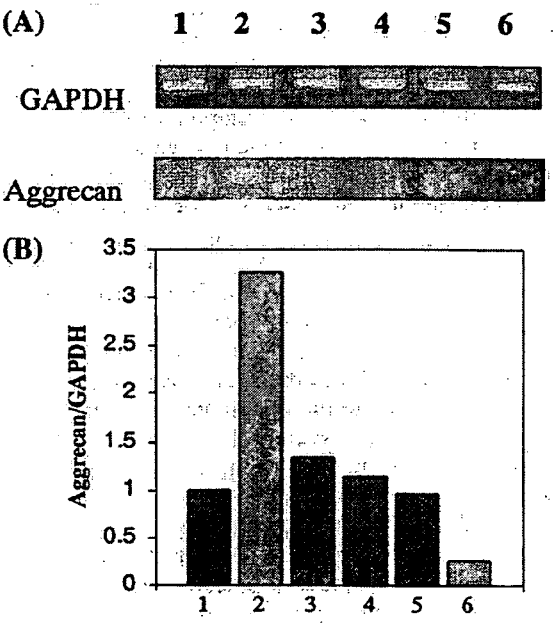

Fig. 5. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of aggrecan gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The mRNA expression of housekeeping gene GAPDH was used for comparing the levels of expression. *Lanes* and *bars* as defined in Fig. 4

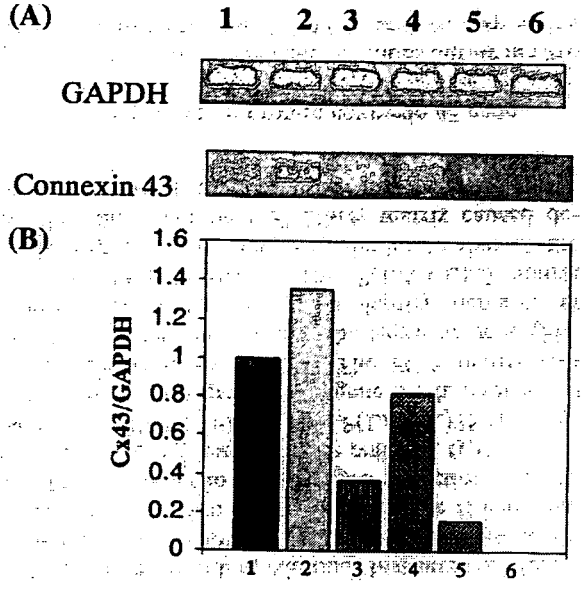

Fig. 6. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of connexin 43 gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable polymers. The expression of GAPDH mRNA was used as an internal control. *Lanes* and *bars* as defined in Fig. 4

NIH image analysis was performed with connexin 43 in 4-week cultured human articular chondrocytes treated with various biodegradable biomaterials. Cx43 expression was normalized by comparison to the expression of GAPDH. Figure 6 shows that PGA induced the highest level of Cx43 mRNA expression, and a decreased level of expression was noted in the PLGA- and PGCL-treated cultures. A faint expression for P(LA-CL)25- and almost zero expression for fullerene C-60 DMA-treated cultures were observed.

Multiple regression analysis

Using multiple regression analysis, the correlation was investigated between cartilage proteoglycan production by the alcian blue method and the three gene expression levels. There was a high correlation between cartilage proteoglycan production and the three gene expression levels (data not shown).

Discussion

The evolution of new biodegradable polymers has drawn much attention in recent years, mainly because of growing application in clinical use. PCL is being utilized for biomedical applications such as controlled drug delivery systems[20] and also as surgical implants in rabbits.[21] Just as for PGA and PLLA, PCL degrades to a naturally occurring metabolite, 6-hydroxyhexanoic acid. To date, research to improve materials and the bioactivity of materials for tissue engineering has centered on PGA and PLLA; however, a short resorption time and low strength characteristics are two major drawbacks of these biodegradable materials. To widen the spectrum of biomaterial choices in tissue engineering, we investigated a copolymer of PGA and PCL, namely, PGCL, and copolymers of PLLA and PCL namely, P(LA-CL)25, PCL(Ti), and fullerene C60 DMA. To compare the bioactivity of these materials with commonly used materials, PGA and PLGA were included in this study. We also included PCL(Sn), synthesized using stannous 2-ethyl hexanoate as the catalyst, in our initial study, but following overnight culture after cell spotting, the cells were detached as a white condensed mass from 15 of 16 PCL(Sn)-coated glass wells in repeated studies. Therefore, PCL(Sn) was excluded from this study. Cells were also detached from 8 (50%) of a total of 16 glass wells coated with PCL(Ti). Thus, both PCL(Ti) and PCL(Sn) matrices were harmful to the cell attachment process. Decreased attachment of human articular chondrocytes with PCL matrix was previously reported.[22] After culture periods of 4 weeks, cell proliferation was significantly inhibited by the PCL(Ti) matrix, and together with its poor cell attachment ability, this ruled out PCL(Ti) as a matrix for future chondrocyte culture. The significant inhibition of cell proliferation by P(LA-CL)25 and fullerene C60 DMA matrices was a result of their narrow range of standard deviation, but, with proliferation levels of 93% of that for the control, they remain feasible candidates for tissue engineering biomaterials. Other matrices had comparable cell proliferation to the control.

During differentiation, chondrocytes secrete extracellular matrix (ECM) molecules characteristic of cartilage, such as type II collagen, aggrecan, and link protein, offering an environment that preserves the chondrocyte phenotype. Therefore, chondrocytes are defined both by their morphology and their ability to produce these characteristic ECM molecules. Collagen type II is regarded as the most important component among the ECM molecules. Previous study detected type II collagen as early as 7 days after beginning 3-D culture, and at 21 days, the matrix of the entire aggregate contained type II collagen.[23] Among the ECM molecules, aggrecan is a major proteoglycan.[24] It has been reported that in chick cartilage, aggrecan expression starts at embryonic day 5 in limb rudiments, continues through the entire period of chondrocyte development, and remains a biochemical marker of the cartilage phenotype thereafter.[25]

In this study, we demonstrated good cell differentiation with the formation of cartilaginous nodules on culture plates by alcian blue staining, which is commonly used for identification of cartilage, and by expression of ECM molecules collagen type II and aggrecan. The morphology after the designated culture period revealed that cells aggregated on the culture plate and formed cartilaginous nodules (Fig. 1). These nodules were first observed after 1 week of culture and progressively became denser as culture continued. These nodules contained copious amounts of ECM, which became stained intensely with alcian blue. The greatest cell

differentiation, a 3.1-fold increase of that of the controls, was found in the sample treated with PGA. The potencies of cell differentiation after 4 weeks of culture from the highest to the lowest were in the following order: PGA >> PLGA > PGCL > Control = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C60 DMA. The increased cell differentiation with PGA and PLGA matrices are in agreement with our previous findings in a micromass culture system;[19] however, in our present study we included the matrix gene expression of these materials. The cell differentiation findings of PCL(Ti) and copolymers PGCL and P(LA-CL)25 could not be compared with other studies because we found no reports describing the effects of PCL and its associated polymers on chondrocyte differentiation. The recent discovery that fullerene C60 DMA can be produced in macroscopic quantities has sparked much interest in the chemistry of this unusual molecule, which did not cause acute toxic effects on mouse skin epidermis.[26] Increased cell proliferation and differentiation of rat embryonic limb bud cells by fullerene C60 were reported,[11] but the data of the present study showed that fullerene C60 DMA acted as a potent inhibitor of HAC differentiation.

As tissue engineering becomes increasingly complex, there is a need to understand how a specific biomaterial influences gene expression. Therefore, the matrices used in this study were evaluated with respect to their influence on the expression of collagen type II and aggrecan genes (Figs. 4 and 5). The increased expression of collagen type II and aggrecan genes in the PGA-, PGCL-, and PLGA-treated matrices was well correlated with their elevated level of cell differentiation values, as shown by alcian blue staining. The low expression of collagen type II and aggrecan genes in the fullerene C60 DMA-treated matrix paralleled the decreased level of cell differentiation, as shown by alcian blue. Therefore, low cell proliferation and differentiation values along with almost no expression of collagen type II and aggrecan genes in the fullerene C60 DMA-treated matrix completely exclude this matrix from use in ECM tissue engineering. The expression of collagen type II and aggrecan genes in the P(LA-CL)25-treated culture was consistent with its cell differentiation value. The data from this study showed that cultured chondrocytes also retained their phenotype throughout the experimental period, as indicated by expression of the type II collagen gene (Fig. 4A, 4B). To the best of our knowledge, this study is the first to show the bioactivity of PCL(Ti) and copolymers PGCL and P(LA-CL)25 in chondrogenic differentiation of HAC in a micromass culture system. Further, we know of no studies that have evaluated the matrix gene expression for PGA and PLGA matrices using HAC in a micromass culture system. Results of the present study confirmed PGA, PLGA, and PGCL as useful scaffolding matrices for cartilage tissue engineering, and information about the other matrices will further contribute to the development of improved cartilaginous constructs for future clinical implants.

The progression of chondrogenic differentiation can be followed by the expression of markers of cytodifferentiation. For example, precartilaginous condensations express type I collagen,[27] whereas the next phase of cartilage differentiation involves the expression of type II collagen, aggrecan, and link proteins, which form the cartilage matrix.[28] The mechanism of precartilaginous condensation is poorly understood, but cell–cell interactions are putative effectors for chondrocyte aggregation.[29] Chondrocytes in the primary culture can proceed through the same differentiation program as they do in the cartilaginous angle of the long bone, and the earliest morphological event on the way to overt differentiation is the formation of cell condensation.[17] The observed expression of Cx43 suggested that the process of condensation is in part caused by the interconnection of cells by means of gap junctions.[13] In this study, RT-PCR analysis showed that the mRNA level of Cx43 gene expression was consistent with chondrogenic differentiation in the presence of different biomaterials. Our findings on Cx43 expression by chondrocytes are in agreement with a previous study that reported expression of functional gap junctions by chondrocytes isolated from adult articular cartilage.[30] Gap junction-mediated intercellular communication is critically involved in the development of cartilage during differentiation.[31]

Conclusions

The analysis of three set of genes, namely collagen type II, aggrecan, and Cx43 was important to evaluate the effect of biodegradable polymers and other types of cartilaginous scaffolds on the chondrogenesis of HAC for tissue engineering.

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References

1. Taylor MS, Daniels AU, Andriano KP, Heller J. Six bioabsorbable polymers: in vitro acute toxicity of accumulated degradation products. J Appl Biomater 1994;5:151–157
2. Nakamura T, Shimizu Y, Okumura N, Matsui T, Hyon SH, Shimamoto T. Tumorigenicity of poly-L-lactide (PLLA) plates compared with medical-grade polyethylene. J Biomed Mater Res 1994;28:17–25
3. Matsusue Y, Yamamuro T, Oka M, Shikinami Y, Hyon SH, Ikada Y. In vitro and in vivo studies on bioabsorbable ultra-high-strength poly (L-lactide) rods. J Biomed Mater Res 1992;26:1553–1567
4. Furukawa T, Matsusue Y, Yasunaga T, Shikinami Y, Okuno M, Nakamura T. Biodegradation behavior of ultra-high-strength hydroxyapatite/poly (L-lactide) composite rods for internal fixation of bone fractures. Biomaterials 2000;21:889–898
5. Hope PG, Williamson DM, Coates CJ, Cole WG. Biodegradable pin fixation of elbow fractures in children. J Bone Joint Surg 1991;73:965–968
6. Kleinschmidt J, Marden L, Kent D, Quigley N, Hollinger J. A multiphase system bone implant for regenerating the calvaria. J Plast Reconstr Surg 1993;91:581–588
7. Hollinger JO. Preliminary report on the osteogenic potential of a biodegradable copolymer of polylactide (PLA) and polyglycolide (PGA). J Biomed Mater Res 1983;17:71–82

8. Tormala P, Pohjonen T, Rokkanen P. Bioabsorbable osteosynthetic implants of ultra-high-strength poly-L-lactide. A clinical study. Int Orthop 1996;20:392–394
9. Evans GR, Brandt K, Widmer MS, Lu L, Meszlenyi RK, Gupta PK, Mikos AG, Hodges J, Williams J, Gurlek A, Nabawi A, Lohman R, Patrick CW Jr. In vivo evaluation of poly (L-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 1999;20:1109–1115
10. Hadlock T, Sundback C, Hunter D, Cheney M, Vacanti JP. A polymer foam conduit seeded with Schwann cells promotes guided peripheral nerve regeneration. Tissue Eng 2000;6:119–127
11. Tsuchiya T, Yamakoshi YN, Miyata N. A novel promoting action of fullerene C60 on the chondrogenesis in rat embryonic limb bud cell culture system. Biochem Biophys Res Commun 1995;206:885–894
12. Tsuchiya T, Oguri I, Yamakoshi YN, Miyata N. Novel harmful effects of [60] fullerene on mouse embryos in vitro and in vivo. FEBS Lett 1996;393:139–145
13. Kumar NM, Gilula NB. The gap junction communication channel. Cell 1996;84:381–388
14. Solursh M, Linsenmeyer TF, Jensen KL. Chondrogenesis from single limb mesenchyme cells. Dev Biol 1982;94:259–262
15. Willecke K, Hennemann H, Dahl E, Jungbluth S, Heynkes R. The diversity of connexin genes encoding gap junctional proteins. Eur J Cell Biol 1991;56:1–7
16. Schwab W, Hofer A, Kasper M. Immunohistochemical distribution of connexin 43 in the cartilage of rats and mice. Histochem J 1998;30:413–419
17. Loty S, Foll C, Forest N, Sautier JM. Association of enhanced expression of gap junctions with in vitro chondrogenic differentiation of rat nasal septal cartilage-released cells following their dedifferentiation and redifferentiation. Arch Oral Biol 2000;45:843–856
18. Okuda K, Hirota M, Hirobe T, Nagano M, Mochizuki M, Mashino T. Synthesis of various water-soluble C60 derivatives and their superoxide-quenching activity. Fullerene Sci Tech 2000;8:89–104
19. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable polymers. Tissue Eng 2001;7:781–790
20. Hombreiro PM, Zinutti C, Lamprecht A, Ubrich N, Astier A, Hoffman M, Bodmeier R, Maincent P. The preparation and evaluation of poly (epsilon-caprolactone) microparticles containing both a lipophilic and a hydrophilic drug. J Control Release 2000;65:429–438
21. Lowry KJ, Hamson KR, Bear L, Peng YB, Calaluce R, Evans ML, Allen WC. Polycaprolactone/glass bioabsorbable implant in a rabbit humerus fracture model J Biomed Mater Res 1997;36:536–541
22. Ishaug-Riley SL, Okun LE, Prado G, Applegate MA, Ratcliffe A. Human articular chondrocyte adhesion and proliferation on synthetic biodegradable polymer films. Biomaterials 1999;20:2245–2256
23. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998;23:265–272
24. Watanabe H, Yamada Y, Kimata K. Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. J Biochem (Tokyo) 1998;124:687–693
25. Schwartz NB, Domowicz M, Krueger RC Jr, Li H, Mangoura D. Brain aggrecan. Perspect Dev Neurobiol 1996;3:291–306
26. Nelson MA, Domann FE, Bowden GT, Hooser SB, Fernando Q, Carter DE. Effects of acute and subchronic exposure of topically applied fullerene extracts on the mouse skin. Toxicol Ind Health 1993;9:623–630
27. Langille RM, Solursh M. Formation of chondrous and osseous tissues in micromass cultures of rat frontonasal and mandibular ectomesenchyme. Differentiation 1990;44:197–206
28. Kosher RA, Gay SW, Kamanitz JR, Kulyk WM, Rodgers BJ, Sai S, Tanaka T, Tanzer ML. Cartilage proteoglycan core protein gene expression during limb cartilage differentiation. Dev Biol 1986;118:112–117
29. Frenz DA, Jaikaria NS, Newman SA. The mechanism of precartilage mesenchymal condensation: a major role for interaction of the cell surface with the amino-terminal heparin-binding domain of fibronectin. Dev Biol 1989;136:97–103
30. Donahue HJ, Guilak F, Vander Molen MA, McLeod KJ, Rubin CT, Grande DA, Brink PR. Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Miner Res 1995;10:1359–1364
31. Coelho CN, Kosher RA. Gap junctional communication during limb cartilage differentiation. Dev Biol 1991;144:47–53

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In vitro study on the osteogenesis of normal human osteoblasts cultured on the discs of various kinds of calcium phosphate ceramics

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Abstract. We estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts. In the present study, five kinds of CP ceramics, namely, hydroxyapatite (HAp) flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), were tested. Cytotoxicity test was carried out using V79 fibroblasts by colony assay system. The amounts differentiation level of NHOst was estimated from alkaline phosphatase (ALP) activity and osteocalcin.
From the results of colony assay, FAp and α-TCP showed strong cytotoxicities on V79 cells. The results from the proliferation studies of NHOst with CP ceramics were consistent with the results of colony assay. In addition, the ALP activities of NHOst with CP ceramics after 1 week culture were significantly suppressed in comparison with that of NHOst alone. The osteocalcin amounts produced from NHOst cultured on β-TCP was the highest among five kinds of CP ceramics.

Introduction

Calcium phosphate (CP) ceramics have been studied to utilize as the scaffolds for repairing bone defects. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) or β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP), can be biologically bonded to natural bones and their porous materials are effective for restoration of bone defects [1]. Fluoroapatite ($Ca_{10}(PO_4)_6F_2$, FAp) has been reported to have a potential of novel bone repairing materials with high stability *in vivo*, since solubility of FAp is lower than that of HAp [2]. In addition, CP cement is also promising for bone repair and it is well known that α-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) or tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement [3].

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical or chemical property, should be satisfied. Among them, biological safety and osteogenesis properties, e.g. proliferation and differentiation of the osteoblasts, should be important factors to provided to the biomaterials. However, understandings of the biological interaction between osteoblasts and various CP ceramics are few, since the interaction has not been studied under the same experimental condition in detail. Therefore, we estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts in this study.

Materials and Methods

Materials

Cytotoxicity and osteogenesis of NHOst on five kinds of CP ceramics, namely, HAp, FAp, α-TCP, β-TCP and TTCP (Wako chem. Co. Ltd., Tokyo, Japan), were evaluated. 0.25g of CP powders was put into stainless mold and uniaxially pressed at 30MPa for 1 min to form pellets. The dimensions of the obtained CP pellet were 1mm in thickness and 12mm in diameter. CP pellets were sterilized by the autoclave 121°C for 20 min.

Cytotoxicty test on CP ceramics

Cytotoxicity test was carried out using Chinese hamster V79 lung fibroblasts by the colony assay system. V79 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd.,) with 10% fetal calf serum (FCS, Intergen company) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The method of cell seeding was shown below; At first, each CP pellets were placed in each culture wells of 24 well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well. Next, 50 cells/300μl of culture medium was added into each well and incubated for 4 h at 37°C. Finally, 400μl of culture medium was added and incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days.

In order to investigate the cell adhesive property on the CP ceramics, the culture medium was changed after cultivations for 4 h and incubated for 7days. The removed culture medium was transferred to another well of the plate and incubated for 7days as well.

Cytotoxicity of extracts from CP ceramics was also investigated in this study. Suspensions of CP ceramics in the culture medium (100mg/mL) were stirred at 37°C for 3days in 150rpm. The suspensions were centrifuged and the supernatants were collected to use as test extracts. The cytotoxicity test was carried out culturing 50 V79 cells in 1ml of the extracts and incubated at 37°C for 7days.

After 7days, the cells were fixed in methanol and the number of the V79 colonies was counted after staining cells with 5%-Giemsa solution. In addition, the pH of the medium after 7-days culture was measured to estimate effect of the pH of the medium on the cytotoxicity test.

Osteogenesis evaluation of NHOst cultured on CP ceramics

NHOst were purchased from BioWhittaker Inc.(Walkersville,MD). The NHOst were maintained in alpha minimum essential medium (αMEM, Gibco, Grand Island, NY) containing 10%-FCS. incubators at 37 °C in a humidified atmosphere with 5% CO_2. All assays were performed using αMEM containing 10%-FCS supplemented with 10mM beta-glycerophosphate. Similar to the method of the cytotoxicity test, each CP pellets were placed in 24-well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well, followed by addition of 1ml of cell suspension (4×10^4 cells/ml) into each well.

Proliferation of NHOst cells cultured on various kinds of CP ceramics was estimated by Tetracolor One assay (Seikagaku Co., Ltd. Tokyo, Japan), which incorporates an oxidation reduction indicator based on detection of metabolic activity. After 7-days incubation, 2%-TetraColor One/αMEM solution was added to each well, followed by 2h incubation. The absorbance of the supernatant at 450nm was estimated using μQuant spectrophotometer (Bio-tek Instrument, Inc., Winooski, VT). After estimating the proliferation, the cells were washed by phosphate–buffered saline (PBS(-)), followed by addition of 1ml of 0.1M glycine buffer (pH=10.5) containing 10mM $MgCl_2$, 0.1mM $ZnCl_2$ and 4mM p-nitrophenylphosphate sodium salt. After incubating at room temperature for 5min, the absorbance of 405 nm of glycine buffer was detected using μQuant spectrophotometer to evaluated alkaline phosphatase (ALP) activity of the test cells. The amount of Osteocalcin produced by NHOst was evaluated using Gla-type Osteocalcin EIT kit (Takara. Co., Ltd.). The structural change of CP before and after autoclave sterilization or culture were investigated by powder X-ray diffraction (XRD) analysis and scanning electron microscopy(SEM). XRD analysis was carried out (Rigaku Co., Ltd. / RINT 2000) with the CuK_α radiation at 40kV, 50mA. SEM observations were performed (JEOL / JSM-5800LV) with an accelerating voltage of 25kV.

Results

Cytotoxicity of various CP ceramics

The results of the cytotoxicity test of CPs are summarized in table 1. Notably, the colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colony formation against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colony formation on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. From these results, it is suggested that V79 cells were viable and adhered on the pellet after for 4h after seeding despite of the type of CP ceramics, irrespective of the type of CP ceramics. On the other hand, the cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicity was almost the same as that of the respective CP pellets themselves (table1).

Proliferation and differentiation of NHOst cultured on CP ceramics

The effects of various kinds of CP ceramics on the osteogenesis of NHOst are represented in table2. The effects of the CPs on proliferation were consistent with those on the colony formation. Similar to the cytotoxicity test, the proliferation of NHOst was inhibited on FAp and α-TCP pellets. ALP activities of NHOst on CP ceramics after 7-days culture were significantly suppressed in comparison with that of NHOst alone. On the other hand, the osteocalcin amounts produced from NHOst were influenced by the type of CP ceramics. NHOst on β-TCP showed the highest Osteocalcin production among five kinds of CP ceramics.

4. Discussion

The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are strongly cytotoxic. It is suggested that the differences in the colony formation on various CP pellets are ascribed to difference in extract properties from the CP related with the composition or crystal structure (table1). In addition, proliferation of NHOst also was inhibited on FAp and α-TCP. The pH values of culture medium after incubation for 7 days are shown in table1. As shown in the table, the pH of culture medium after incubation with FAp pellets is almost the same as that of HAp, while the pH of the α-TCP medium is much lower than other CP ceramics. In order to considering the reason of the low pH of the culture medium with α-TCP pellet, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP after extraction treatment are shown in Fig.1.

Table1. Cytotoxicity test of various CP ceramics.

Samples	Composition	Formation of colony		pH of medium after culturing	Ca concentration[1] /ppm
		On pellets /%	Extraction / %		
V79 alone	-	100.0±4.5	100.0±13.9	7.12	-
HAp	$Ca_{10}(PO_4)_6(OH)_2$	58.1±12.8	84.6±15.1	7.24	0.19
FAp	$Ca_{10}(PO_4)_6F_2$	22.6±20.9**	26.9±8.6*	7.20	0.17
α-TCP	α-$Ca_3(PO_4)_2$	0.0*	7.6±5.1*	6.76	72.62
β-TCP	β-$Ca_3(PO_4)_2$	57.3±6.9	81.1±19.3	7.40	1.27
TTCP	$Ca_4(PO_4)_2O$	78.2±5.0	93.7±6.8	7.65	0.58

*p<0.01 against V79 alone, **p<0.05 against V79 alone, 1)The Ca ions concentration was extracted Ca ions from CP-ceramics in PBS(-), which were measured by inductivity coupled plasma-atomic emission spectroscopy.

Table2. Osteogenesis of NHOst cultured on various kinds of CP ceramics.

Samples	Proliferation / %	Differentiation level	
		ALP activity / %	Osteocalcin / %
NHOst alone	100±7.9	100±4.4	100±46.2
HAp	63.2±3.5	20.4±1.8*	81.1±31.0
FAp	42.9±19.5	1.24±0.3*	47.2±20.7
α-TCP	18.3*±2.5	17.9±3.8*	110.7±18.8
β-TCP	56.0±4.7	6.3±3.2*	177.1±78.4**
TTCP	82.3±27.3	17.5±4.6*	114.8±4.0

*p<0.01 against NHOst alone, **p<0.01 against HAp

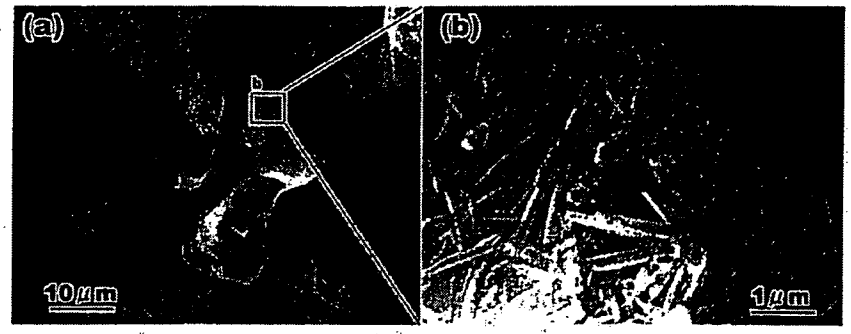

Fig.1. SEM images of α-TCP after extract treatment. (a) whole image and (b) enlarged image of the area enclosed by a rectangle in (a)

Before the extraction, a particle size of α-TCP was about 10μm and its surface was smooth. However, as shown in Fig.1, whisker-like precipitates of 1-2μm in length and 2-300nm in width are observed at the surface of α-TCP after the extraction, although there is no change in its particle size. It is well known that slightly water-soluble calcium phosphates convert to HAp in aqueous solution with high pH value. Since the solubility of α-TCP is higher than that of other calcium phosphates, the α-TCP converts to HAp rapidly as following the reaction.

$$10Ca_3(PO_4)_2+6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2+2H_3PO_4$$

According to the report of this conversion [4], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation. In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid caused the decrease in the pH of solution. Morita and co-workers [5] have reported that low pH itself could be clastogenic to mammalian cells and the pH of 50% V79 cell survival was 6.5 for 24h incubation. Therefore, it is suggested that the cytotoxicity of α-TCP was mainly due to the pH decreasing resulting from an increase of the phosphoric acid ion produced by the hydrolysis conversion from α-TCP to HAp.

On the other hand, FAp has the same crystal structure of HAp but the hydroxyl ions in HAp substituted by fluorine ions. Since difference of the colony formation on various CP ceramics would be due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp.

Effects of CP ceramics on osteogenesis function of NHOst are shown in table2. As shown in the table, ALP activities of NHOst were significantly suppressed on CP ceramics irrespective of their type and the amount of osteocalcin on β-TCP was the highest among five kinds of CP ceramics. Since it is well known that osteocalcin express in maturated stage of differentiation level of NHOst, these results suggest that maturation of NHOst proceeds on β-TCP. . The differences of maturation of NHOst on various kinds of CP ceramics may be related with the amount of extracted Ca^{2+} and/or PO_4^{3-} ions.

References

[1] Y. Ito, N. Tanaka, Y. Fujimoto, Y. Yasunaga, O. Ishida, M. Agung, M. and Ochi, J. Biomed. Mater. Res: Vol.69A(3) (2004) p454

[2] K. Cheng K, W. Weng, H. Qu, P. Du, G. Shen, G. Han, J. Yang and M. J. Ferreira: J.Biomed. Mater. Res. B, Vol.69(1) (2004) p33

[3] M. E. Ooms, J. G. C. Wolke, J. C. P. M. Waerden and J. A. Jansen, Trabecular, J.Biomed. Mater. Res. Vol.61 (2002) p9

[4] M.Tamai, T.Isshiki, K.Nishio, M.Nakamura, A.Nakahira and H.Endoh: J. Mater. Res. Vol.18 (2003), p.2633

[5] T.Morita, T.Nagaki, I.Fukuda and K.Okumura: Muta. Res. Vol.268 (1992), p.297