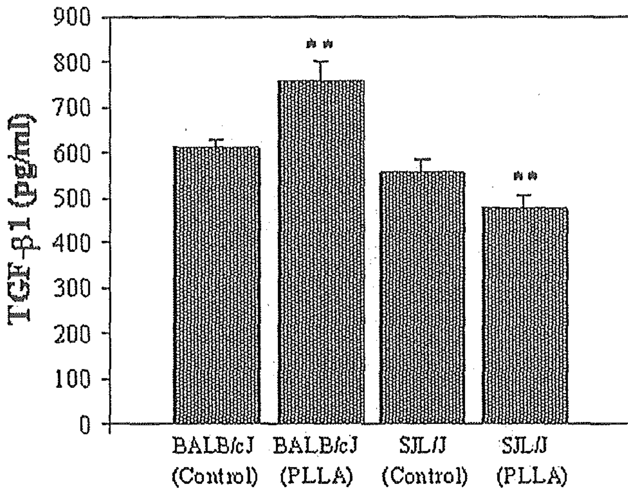

Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Secretion of TGF-β1 level significantly increased in PLLA-implanted BALB/cJ mice when compared with that in BALB/cJ controls. On the contrary, in the SJL/J mice, secretion of TGF-β1 tended to decrease in PLLA-implanted mice when compared with that in control mice. $^{**}p < 0.01$.

Histopathology

Tumor cells from nude mice injected with PLLA-implanted BALB/cJ mouse cells showed monophasic fibrous synovial sarcoma on H&E and keratin AE1/AE3 staining. Tumor cells with a staghorn pattern [Fig. 7(A)] and a herringbone pattern were identified [Fig. 7(B,C)].

DISCUSSION

Polylactides are bioabsorbable polyesters with wide range of clinical applications. Because it degrades slowly, PLLA has been used as a biomaterial for surgical devices such as bone plates, pins, and screws. It has been reported in different studies that polyetherurethane, nonabsorbable polyethylene, and PLLA produced tumors in rats.[9,10,25–27] Parallel to these studies, here cells with different morphologies formed a crisscross pattern, which thus decreased the contact inhibition in the PLLA-implanted BALB/cJ group [Fig. 1(B)]. We examined the protein expression of Cx 43 to evaluate the actual cause and found that

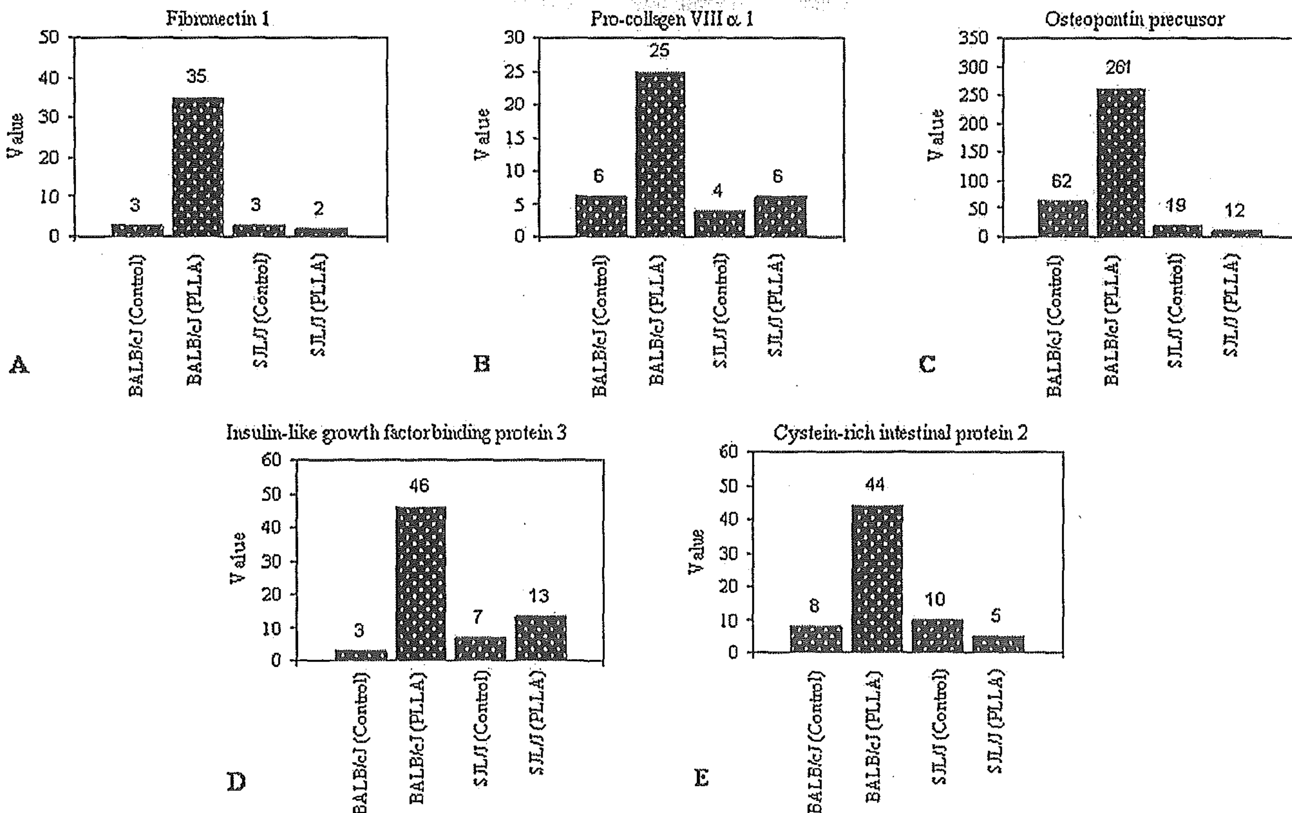

Figure 5. DNA microarray analysis of these four kinds of cells. The expression of (A) fibronectin 1, (B) pro-collagen VIIIα 1, (C) osteopontin precursor (OPN), (D) insulin-like growth factor binding protein (IGFBP) 3, and (E) cysteine-rich intestinal protein 2 (CRIP 2) increased in the cells of PLLA-implanted BALB/cJ mice. Results shown are representative of four independent experiments.

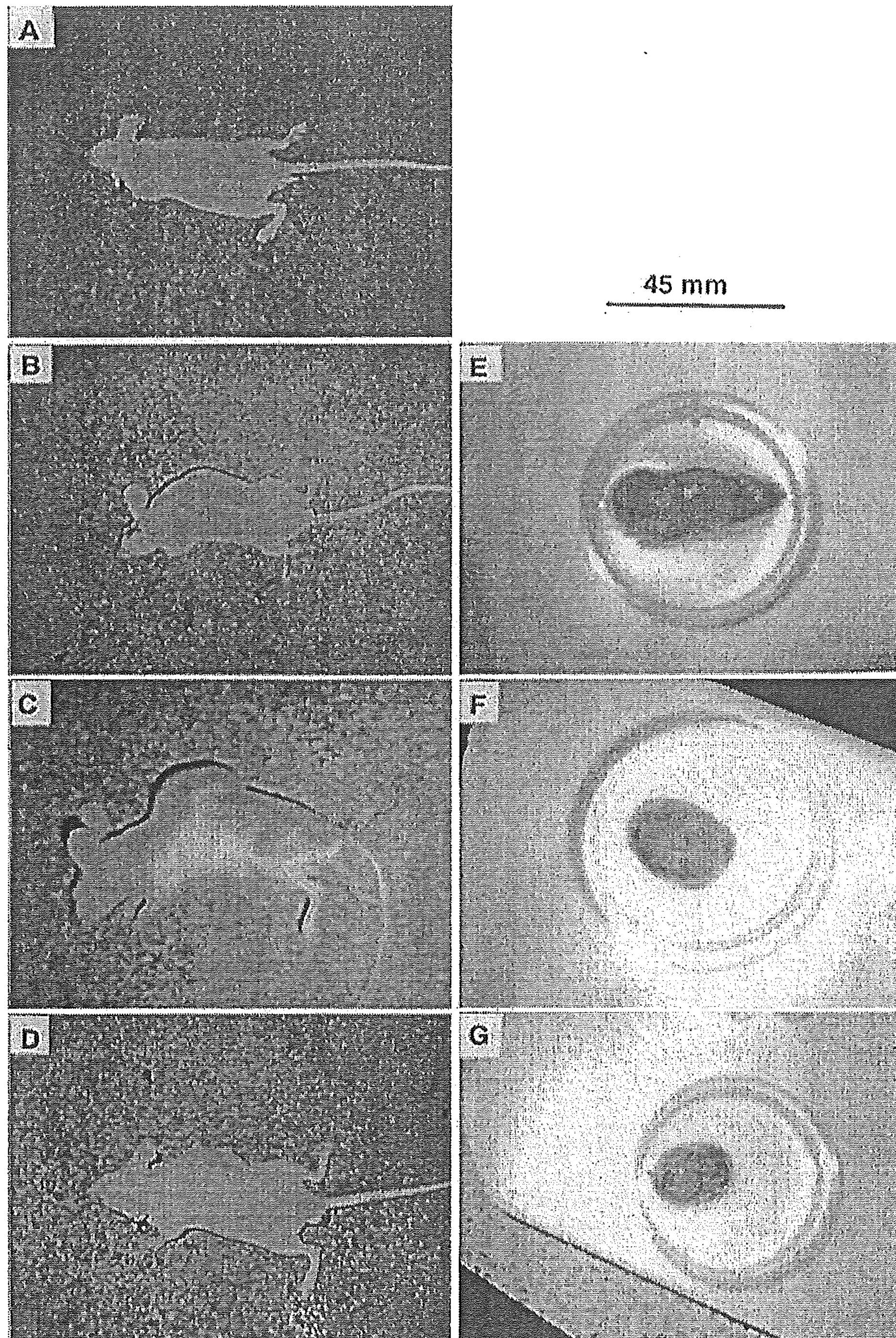

Figure 6. Determination of tumorigenicity in nude mice. (A) No tumor was formed in PBS(−) injected nude mice. (B, C, E, and F) A large tumor growth was observed within two weeks in nude mice injected with cells from PLLA-implanted BALB/cJ mice. (D and G) Tumor growth was observed in nude mice 4 weeks after they were injected with HeLa cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the total level of protein expression was significantly decreased in the PLLA-implanted groups when compared with that in the controls (Fig. 2). In contrast, Cx 43 protein expression decreased in both control and PLLA-implanted groups in SJL/J mice (Fig. 2). We also examined the functional effects on GJIC. In the present study and correlating with our previous report,[22] GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice when compared with that in controls (Fig. 3). Gap junctions are regulated by the posttranslational phosphorylation of the carboxy-terminal tail region on the Cx molecule, and hyperphos-

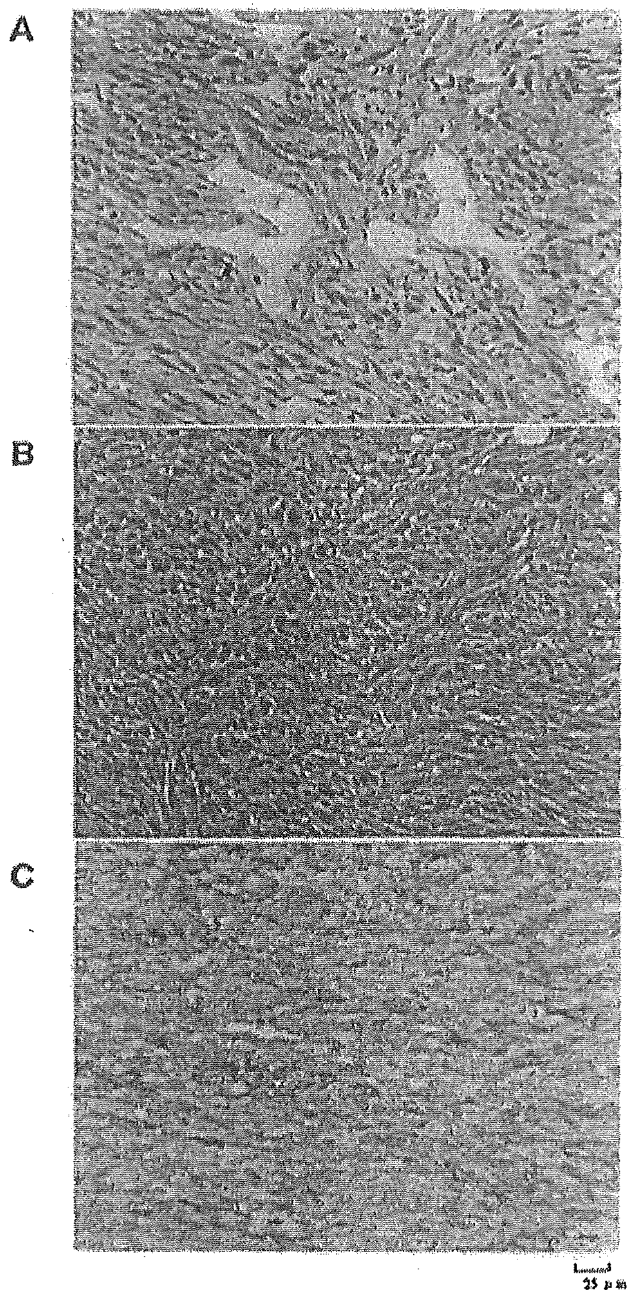

Figure 7. Histopathology. Tumor cells from nude mice injected with cells from PLLA-implanted BALB/cJ mice showed monophasic fibrous synovial sarcoma with H&E and keratin AE1/AE3 staining. (A) Staghorn pattern (H&E), (B) herringbone pattern (H&E), and (C) herringbone pattern (keratin AE1/AE3 staining). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phorylation of Cx molecules is closely related to the inhibition of GJIC.[28,29] Asamoto et al. reported that tumorigenicity enhanced when the expression of Cx 43 protein was suppressed by the antisense RNA of Cx 43.[30] Thus, in our experiment, the impaired GJIC was possibly caused by the suppression of protein expression of Cx 43. Therefore, it is suggested that gap junctions are likely to play a major role in the PLLA-induced tumorigenesis in BALB/cJ mice. But in SJL/J mice, this is not the key factor for tumorigenesis. Another protein may be responsible because Cx 43 protein expression was decreased in both control and PLLA-implanted group of SJL/J mice.

TGF-β1 can impair GJIC function by decreasing the phosphorylated form of Cx 43 (Ref. 31) and can also increase the expression of ECM.[32,33] We estimated the production of TGF-β1 in four kinds of cells. The secretion of TGF-β1 significantly increased in PLLA-implanted BALB/cJ mice cells in comparison with that from BALB/cJ control mice, but TGF-β1 secretion decreased in the SJL/J-implanted group when compared with that in the SJL/J control mice (Fig. 4). Furthermore, by using DNA microarray analysis of these four kinds of cells, expression of the major ECM proteins (fibronectin 1, pro-collagen VIIIα 1, and OPN) and IGFBP 3 was found to be increased in the PLLA-implanted BALB/cJ mice cells (Fig. 5). Several reports have suggested that these proteins could directly cause tumorigenesis.[34–36] Overexpression of CRIP 2, a member of the LIM (characterized by a repeat of a double zinc finger cysteine-rich sequence, CCHC and CCCC) protein family, caused an increase in Th2 cytokine IL-6,[37] and synovial sarcoma cells are reported to produce IL-6 by themselves.[38] Figure 5 shows that IGFBP 3 was highly expressed in the PLLA-implanted BALB/cJ mice cells. In addition, overexpression of IGFBP 3 was associated with poorer prognosis in breast cancer.[36] Therefore, we speculated that overexpression of IGFBP 3 and major ECM proteins directly or indirectly causes tumorigenesis in the PLLA-implanted BALB/cJ mice.

Ten months after implantation of the PLLA plate into BALB/cJ mice, formation of a tissue growth was observed at the implanted site. To determine whether this tissue growth was a tumor or a result of foreign body (PLLA) inflammation, we performed a tumorigenicity assay in nude mice. Rapid growth of a large tumor was observed in nude mice injected with cells obtained from PLLA-implanted BALB/cJ mice (Fig. 6). The histopathologic examination of this tumor disclosed monophasic fibrous synovial sarcoma (Fig. 7). Nude mice injected with HeLa cells as a positive control showed slower tumor growth. However, these PLLA-derived tumor cells did not form a colony in a soft agar assay (data not shown).

We speculated that a protein or regulatory factor other than Cx 43 may play key role in tumorigenesis in PLLA-implanted BALB/cJ mice. In this light, we conclude that overexpression of the regulatory factors such as TGF-β1 and IGFBP 3 caused tumorigenesis in PLLA-implanted BALB/cJ mice. In addition, increased secretion of TGF-β1 suppressed the expression of Cx 43 and inhibited GJIC. Moreover, PLLA

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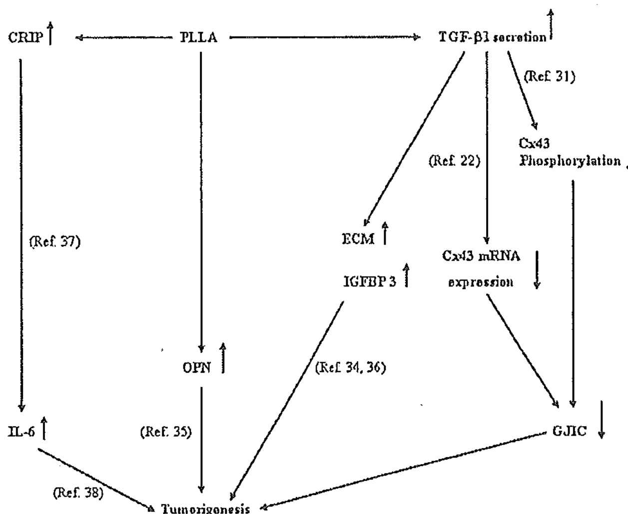

Figure 8. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

increased the expression of ECM, CRIP 2, and OPN. Finally, all these factors in combination promoted tumorigenesis (Fig. 8).

F8

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Author Proof

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

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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]

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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.

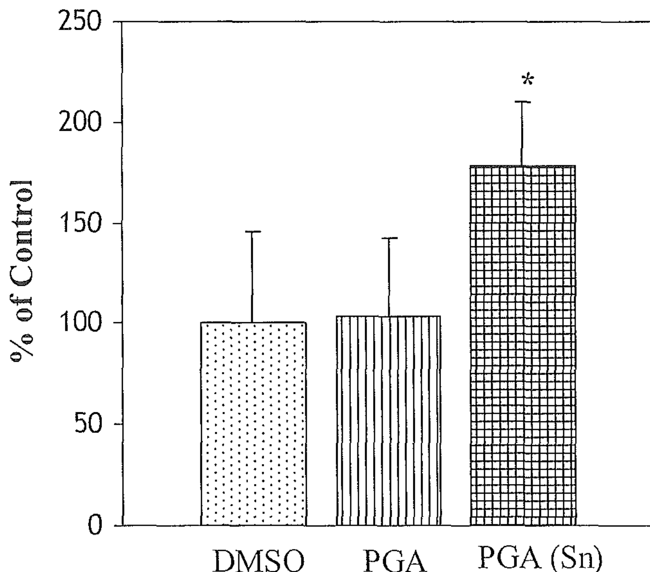

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. $*p < 0.05$. All experiments were run in quadruplicate for two separate times.

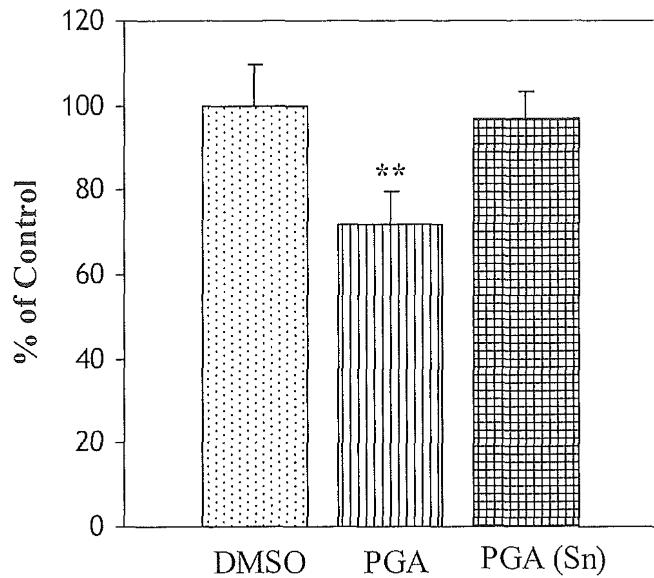

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. $**p < 0.01$. All experiments were run in quadruplicate for two separate times.

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)].

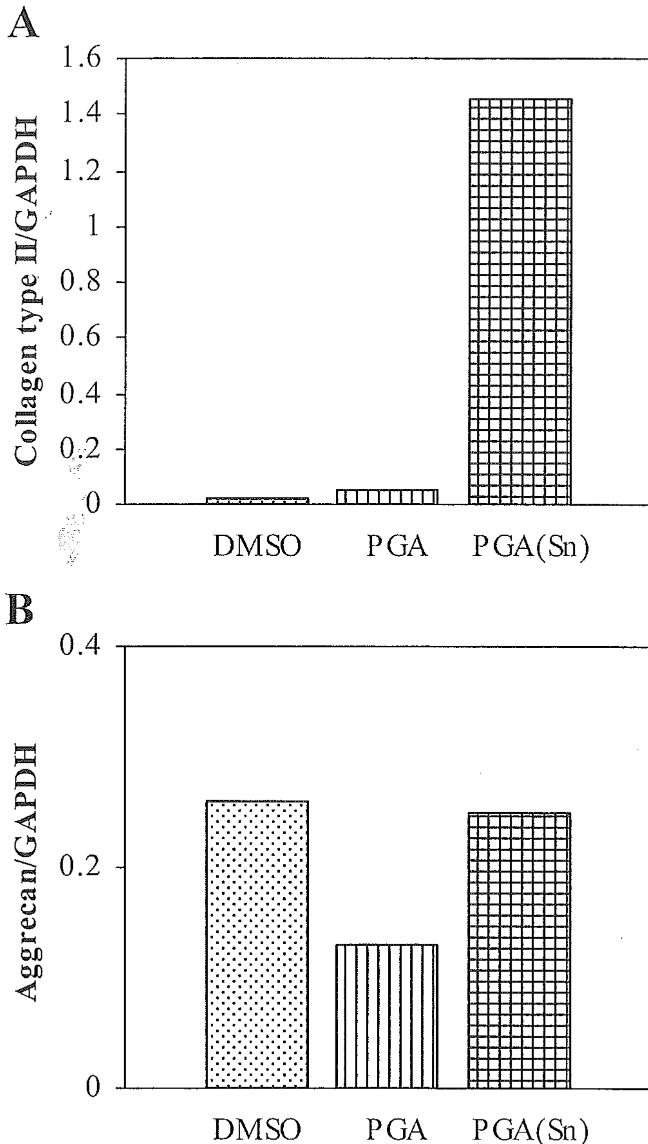

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between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

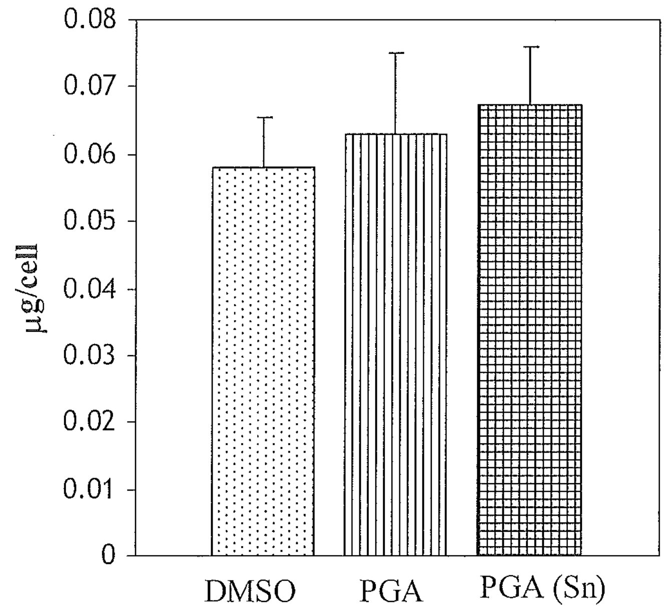

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

Estimation of sulfated glycosaminoglycan concentration

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

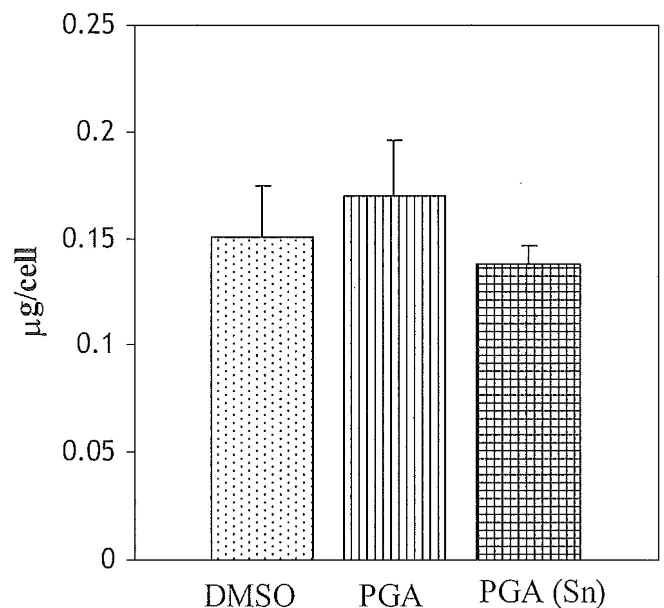

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

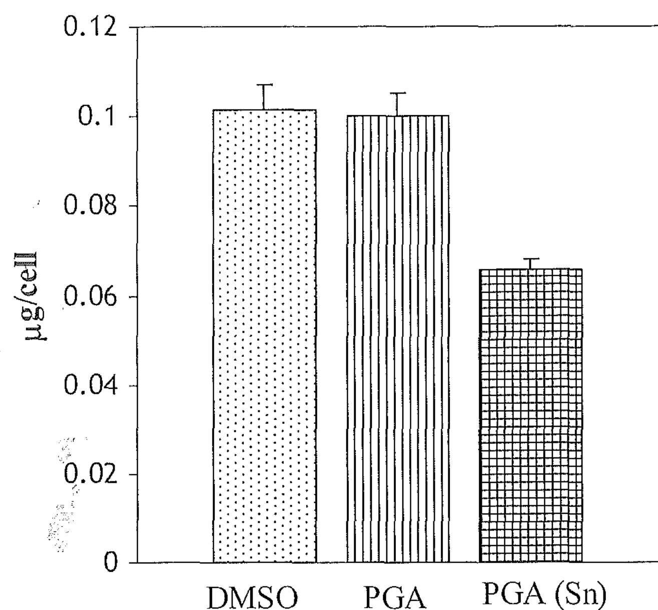

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

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.

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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.

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

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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) 7.5g and caprolactone (Wako Pure Chemical Industries, Osaka, Japan) 2.5g were put into a reactor as monomers. As a catalyst, tetrabutoxy titanium (Wako) 0.03g was added. Furthermore, *n*-octyl alcohol (Wako) 0.001g was added. These were completely dissolved in methylene chloride (Wako) 50mL 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 4h. After the reaction, the reactant was cooled to room temperature, and was dissolved in tetrahydrofuran 100mL. 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% (5.82g).

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% (8.71g).

Preparation of materials

PGA, PGCL, PLGA, and P(LA-CL)25 were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 50μg/0.8μl of DMSO (Sigma-Aldrich, Irvine, CA, USA) and then dissolved in chondrocyte growth medium to give a final concentration of 50μg/ml. PCL(Ti) was dissolved in tetrahydrofran (THF) at a concentration of 5mg of PCL/ml of THF. Glass wells were coated with this solution to give a final concentration of 2mg 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 20μl 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 22mm). After 2h in a 5% CO_2 incubator at 37°C, the wells were flooded with chondrocyte growth medium (2ml/well). The medium was supplemented with DMSO (0.8μl/ml), PGA (50μg/ml), PGCL (50μg/ml), PLGA (50μg/ml), P(LA-CL)25 (50μg/ml), or fullerene C60 DMA (50μg/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 100 μl 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 535 nm for excitation and 590 nm 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 (0.5 ml/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 600 nm 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 0.5 ml 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 260 nm.

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 1 μg 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 4 μg of cDNA in a 20-μl reaction mixture (10 × PCR buffer 2 μl, dNTP 1.6 μl, forward and reverse primer 0.4 μl, Taq DNA polymerase 0.1 μl, and distilled water to make up 20 μl). 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′-ATGGGTGACTGGAGCGCCTTAGGCAA ACTC-3′
reverse 5′-GACCTCGGCCTGATGACCTGGAGATC TAG-3′

For collagen type II and Cx43, an initial denaturation step at 94°C was carried out for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. For aggrecan, an initial denaturation at 95°C was carried out for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min), with a final extension at 72°C for 5 min. 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′-TGGCCAAGGTCATCCATGACAACTTTG G-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.

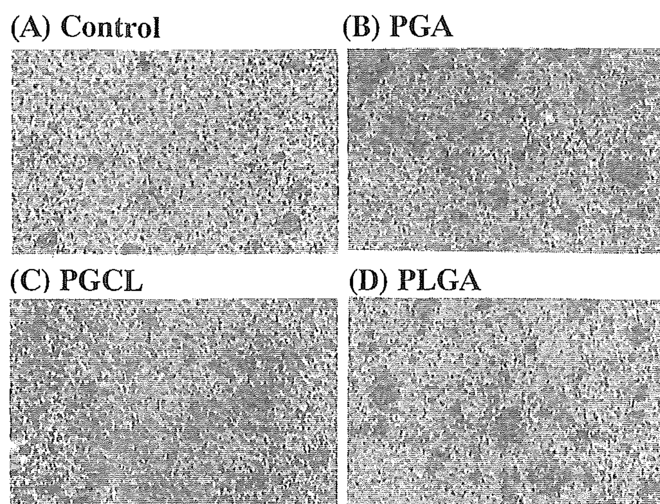

Fig. 1A–D. Light microscopic appearance of cultured human articular chondrocytes spotted as a high-density micromass culture with different biodegradable polymers for 4 weeks. **A** Control, **B** polyglycolic acid (PGA), **C** poly(glycolic acid-ε-caprolactone) (PGCL), **D** poly(L-lactic acid-glycolic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

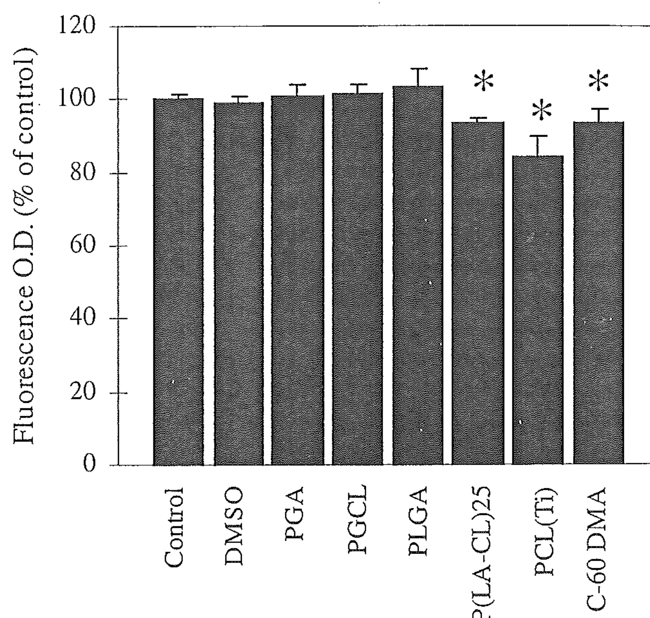

Fig. 2. Cell proliferation of human articular chondrocytes as determined by alamar blue assay after culturing with synthetic biodegradable polymers for 4 weeks. The proliferation in all samples exposed to dimethyl sulfoxide (*DMSO*) and biomaterials were calculated as a percentage of control values. *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 2h 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

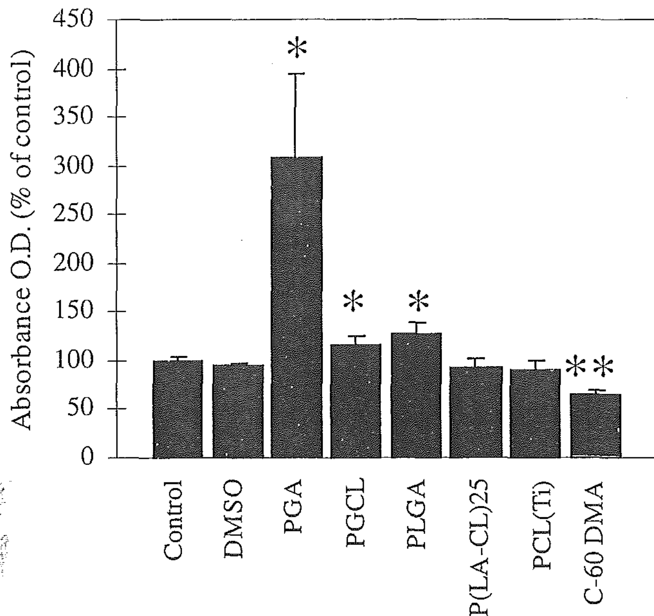

Fig. 3. Cartilage proteoglycan content of human articular chondrocytes as determined by the alcian blue staining method after culturing with synthetic biodegradable polymers for 4 weeks. The values are expressed as a percentage of control values. $^{*}P < 0.05$ and $^{**}P < 0.01$

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

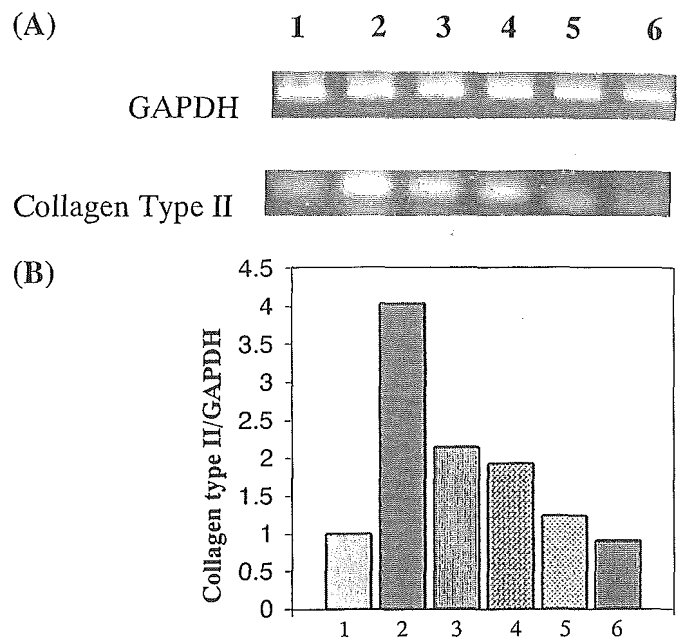

Fig. 4. Reverse transcription polymerase chain reaction (RT-PCR) analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of collagen type-II gene expression was represented by the mRNA level of 4-week cultured human articular 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

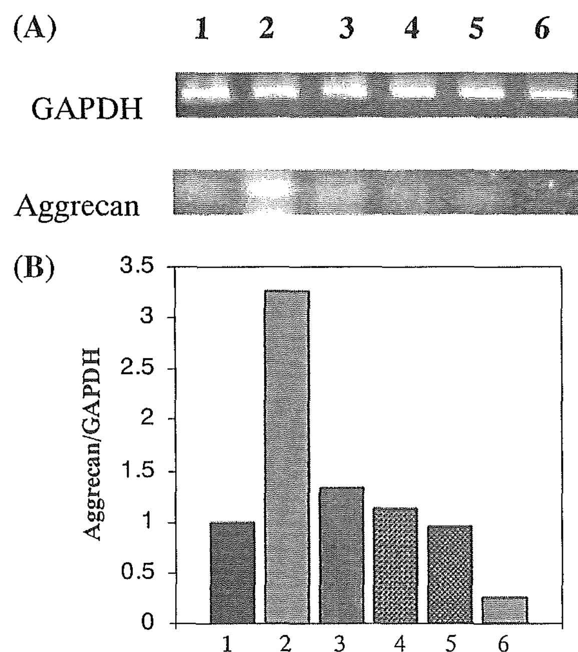

Fig. 5. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of aggrecan gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable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

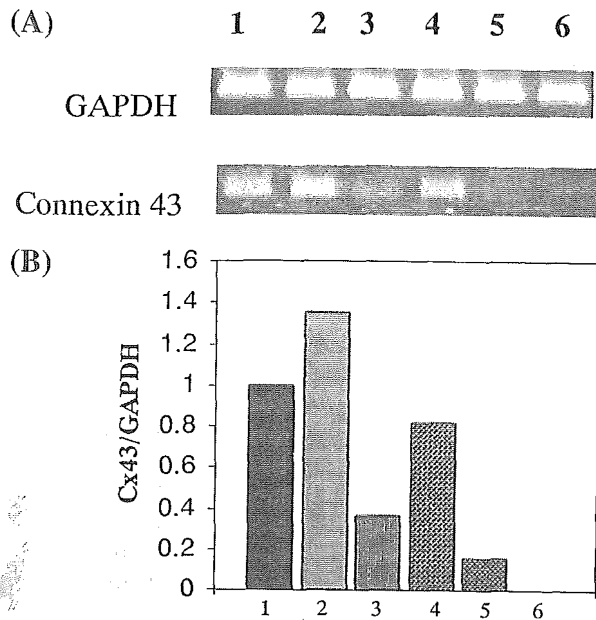

Fig. 6. RT-PCR analysis (**A**) and National Institutes of Health (NIH) image analysis quantitation of RT-PCR bands (**B**). In both figures, the level of connexin 43 gene expression was represented by the mRNA level of 4-week cultured human articular chondrocytes treated with different types of biodegradable 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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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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