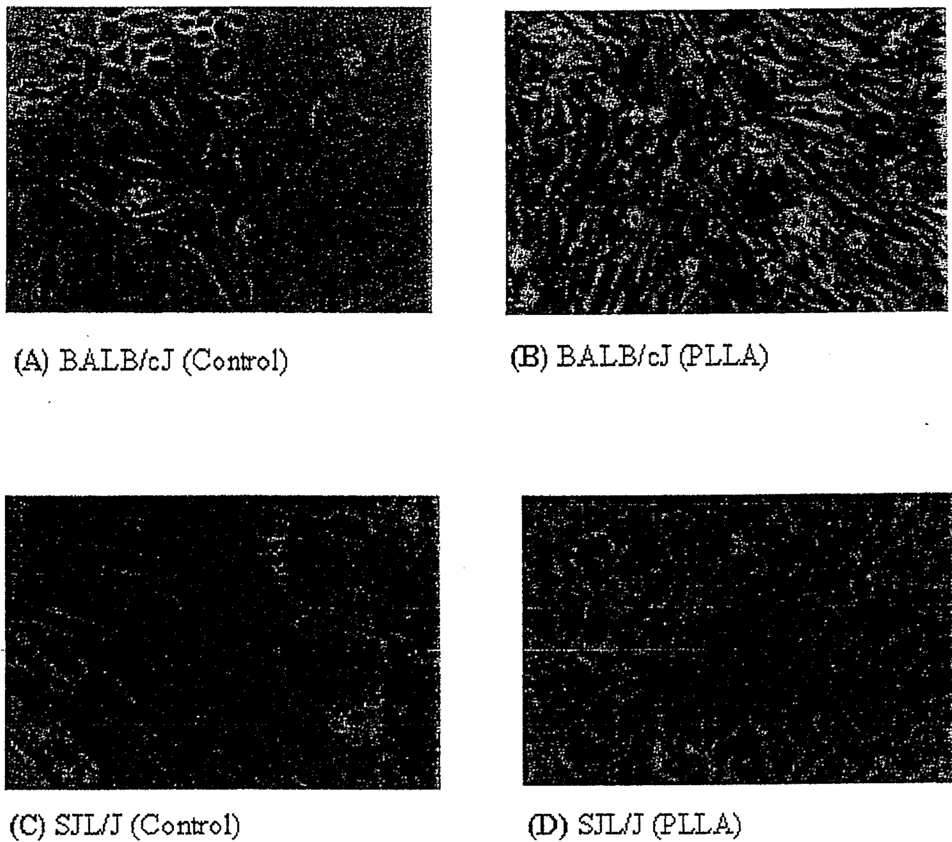

Figure 1. Mouse cell morphology. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Inverted light microscopic appearance (magnification ×100) of (A) BALB/cJ (control), (B) BALB/cJ (PLLA), (C) SJL/J (control), and (D) SJL/J (PLLA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Soft agar assay

These tumor cells did not form a colony in soft agar (data not shown), although HeLa cells did form colonies in soft agar.

Histopathology

Tumor cells from nude mice injected with PLLA-implanted BALB/cJ mouse cells showed monophasic

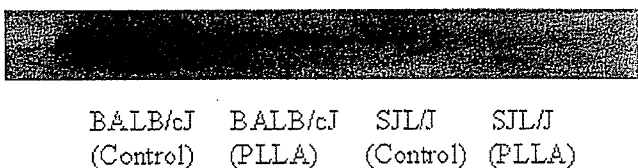

Figure 2. Expression of Cx 43 protein by Western blot analysis. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Total protein expression was significantly decreased in PLLA-implanted BALB/cJ mice when compared with that in the control. However, protein expression was decreased in both control and PLLA-implanted groups in SJL/J mice.

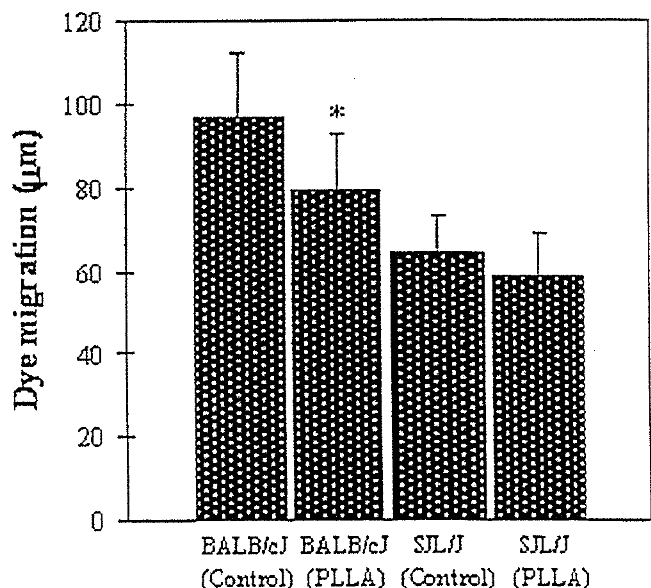

Figure 3. Statistical analysis of SLDT assay. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. GJIC was found to be significantly inhibited in PLLA-implanted BALB/cJ mice cells when compared with that in BALB/cJ controls. $^*p < 0.05$.

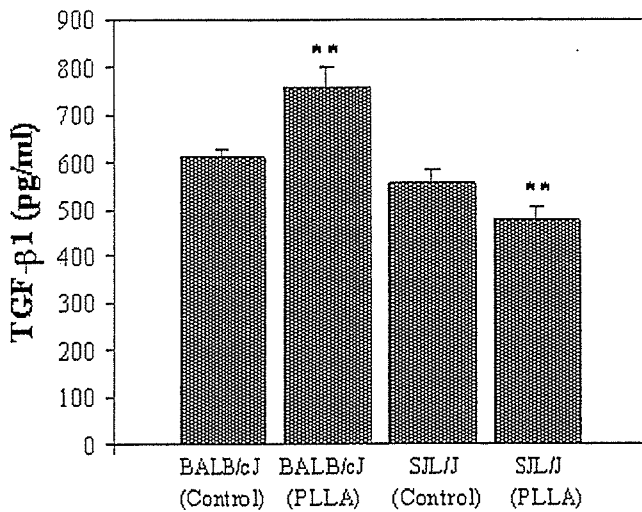

Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Secretion of TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ 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. $^{**}p < 0.01$.

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 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 was decreased in both control

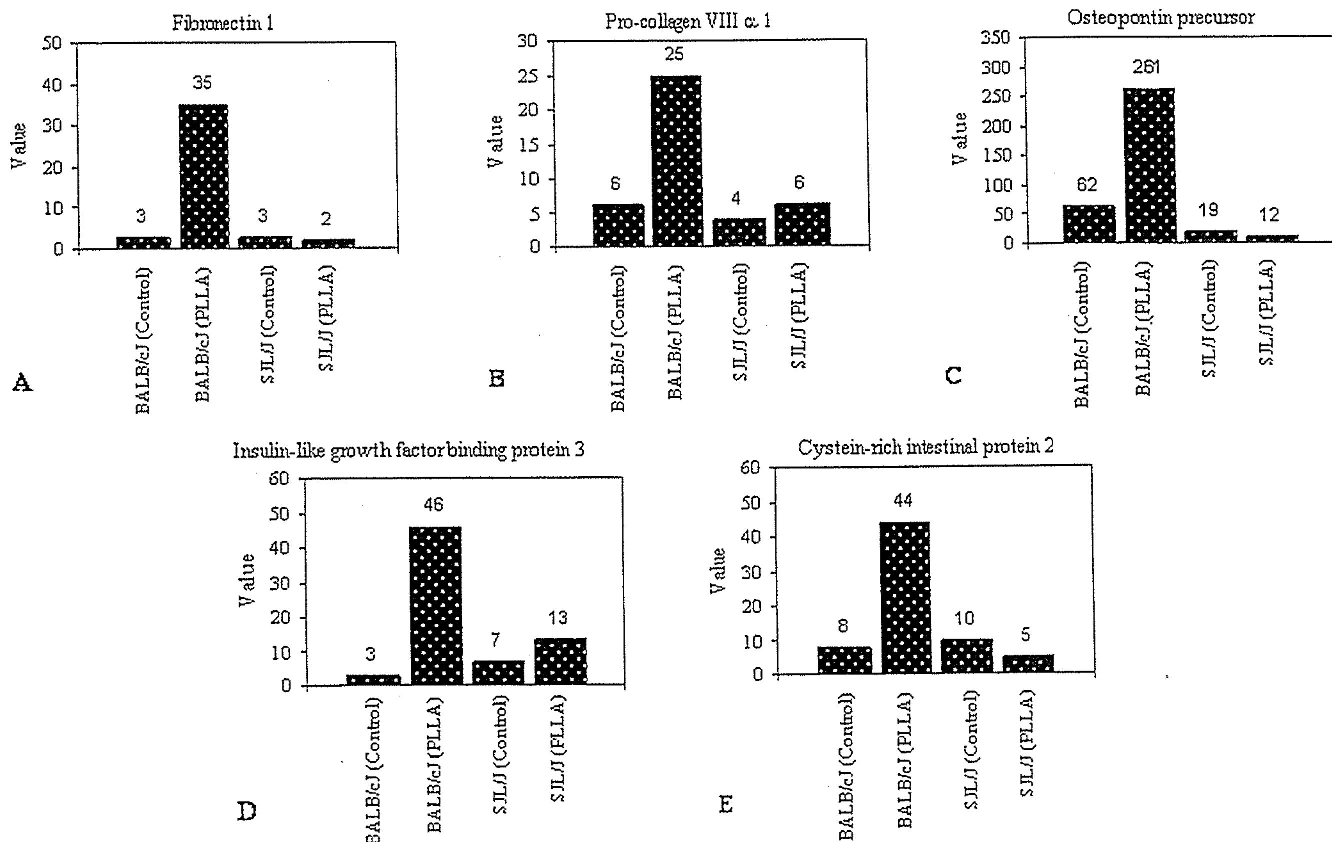

Figure 5. DNA microarray analysis of these four kinds of cells. The expression of (A) fibronectin 1, (B) pro-collagen VIIIα 1, (C) osteopontin precursor (OPN), (D) insulin-like growth factor binding protein (IGFBP) 3, and (E) cysteine-rich intestinal protein 2 (CRIP 2) increased in the cells of PLLA-implanted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

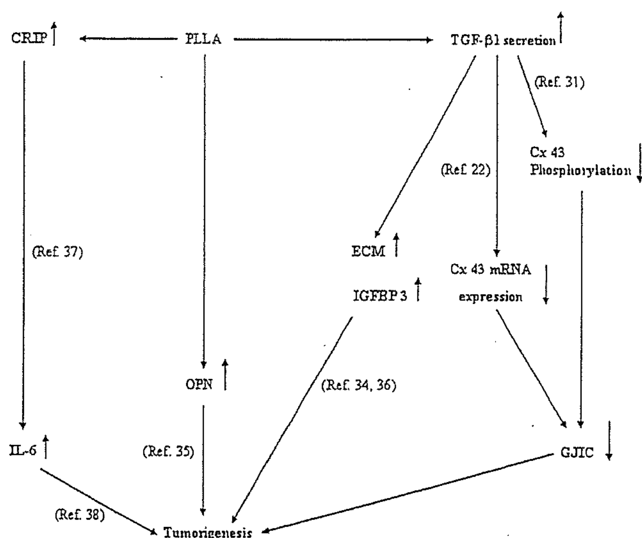

Figure 8. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

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STUDIES ON THE EFFICACY, SAFETY AND QUALITY OF THE TISSUE ENGINEERED PRODUCTS: ENHANCEMENT OF PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS BY THE NEW POLYSACCHARIDES

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Abstract: Human mesenchymal stem cells (hMSCs) have the capacity to proliferate and differentiate into multiple cells etc. Polysaccharides can modulate the cell proliferation of human endothelial cell. Here, we investigated the role of different kinds of new polysaccharides to regulate the gap junctional intercellular communication (GJIC) and cell proliferation of cultured normal human dermal fibroblasts (NHDF) cells and hMSCs. The NHDF cells and hMSCs were cultured for 4 days with new polysaccharides. The cultures were then analyzed to verify the extent of GJIC by the scrape-loading dye transfer (SLDT) method, using Lucifer yellow. Alamar blue staining was performed to determine the proliferation of the cultured cells. In NHDF cells, the GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides. On the contrary, in hMSCs, the GJIC was slightly inhibited in all cultured treated cells. But proliferation was enhanced in both cells with different polysaccharides, the extents of cell proliferation was stronger in hMSCs than in NHDF cells. These findings reveal that new polysaccharides seem to play an important role in hMSCs, thus provide a novel tool on tissue engineering.

Key words: GJIC, Proliferation, NHDF, hMSCs.

1. INTRODUCTION

Human mesenchymal stem cells (hMSCs) are multipotent cells have the capacity to proliferate and differentiate into bone, cartilage and adipocytes, and are useful for human cell and gene therapies [1]. Polysaccharides are macromolecules formed from many sugar units connected by glycosidic

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linkages. It has two basic functions: serve for monosaccharide storage to make cellular energy and serve as structural components. Sulfated polysaccharide was reported to cause modulation of human endothelial cell proliferation [2]. Sweeney *et al.* also reported that sulfated polysaccharide increases and mobilizes hematopoetic stem cells in mice and nonhuman primates [3]. Furthermore, the inhibition of GJIC can disrupt the balance of cell homeostasis, leading to increase cell proliferation [4]. The aim of this study is to investigate the ability of different kinds of new polysaccharides to regulate the GJIC and cell growth of cultured NHDF cells and hMSCs.

2. MATERIALS AND METHODS

2.1. ***Materials:*** 4 different kinds of polysaccharides were used in this experiment.

2.2. ***Cell Culture:*** The NHDF cells were obtained from Asahi Techno Glass (Tokyo, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5 % CO_2 atmosphere at 37°C. The hMSCs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, USA), and maintained in mesenchymal cell growth medium (MSCGM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C.

2.3. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Cells $1x10^5$/ml (2ml medium/dish) were seeded on to the 35 mm dishes. After 4 hr seeding in a 5% CO_2 atmosphere at 37°C, different kinds of new polysaccharides at the concentration of 2mg/ml, 1ml per dish (35mm dish) was added and incubated at 37°C for 4 days. Then, confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS (+) and the extent of dye migration length was measured using fluorescence microscope.

2.4. ***Proliferation assay:*** $4x10^4$ (0.5 ml medium/well) cells per well of 24 well culture plate were seeded. After 4 hr seeding in a 5% CO_2 atmosphere at 37°C, different kinds of new polysaccharides at the concentration of 2mg/ml was added and incubated at 37°C for 4 days. Then, cell proliferation was quantitatively measured by alamar blue (Biosource International, Inc., Camarillo, CA) assay. The assay showed the metabolic activity of the cells by detection of mitochondrial activity. Here, alamar blue used as the indicator dye, was incorporated into the cells, reduced and excreted as a fluorescent product. At the end of 4 days culture, the media from all wells were discarded, and filled with 1 ml/well of 1:20 of alamar blue/fresh medium. The culture plates were incubated at 37°C for 4 h. After the incubation period, two aliquots of 100 μl of solution from each well were transferred into new wells of a Costar 96-well

microplate of tissue culture (Costar type 3595, Corning Co. Ltd.). Equal volume of fresh medium per well (total four wells) served as blanks. The extent of cell proliferation was quantitated by Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535-nm excitation and 590-nm emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from the experimental values to eliminate background readings.

2.5. *Statistical analysis:* Student's t test was used to compare the implanted samples with the controls. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean±SD.

3. RESULTS

NHDF cells: In NHDF cells, GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides (**p < 0.01) (Figure 1A). But the cell proliferation was significantly increased in cells treated with different kinds of polysaccharides (**p < 0.01) (Figure 1B).

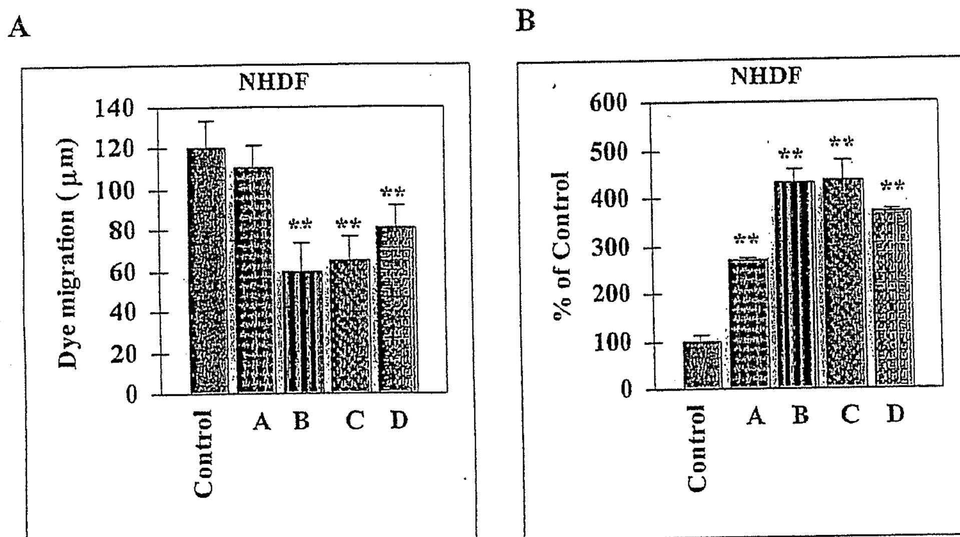

Figure 1. In A, Statistical analysis of SLDT assay and in B, cell proliferation of NHDF cells. ** p < 0.01.

hMSCs: In hMSCs, GJIC was also inhibited in all treated cells but significantly in only treated with "D" (*p < 0.05, **p < 0.01) (Figure 2A). Here proliferation also was significantly enhanced in cells treated with different kinds of polysaccharides (**p < 0.01) (Figure 2B). But stimulatory reaction was much more in hMSC cell than NHDF cell.

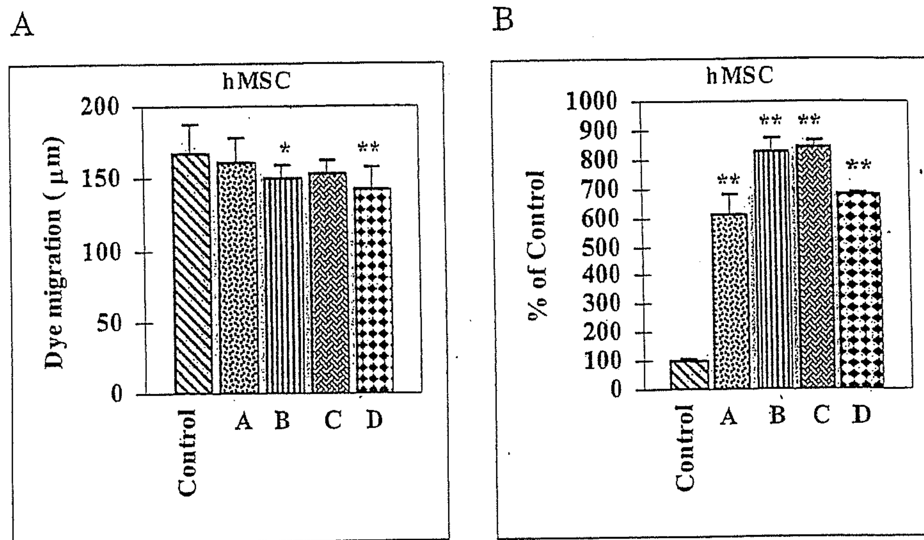

Figure 2. In A, Statistical analysis of SLDT assay and in B, cell proliferation of hMSCs. * $p < 0.05$, ** $p < 0.01$.

4. DISCUSSION

hMSCs are used for tissue engineering of bone and cartilage and provide a versatile model system to study mesenchymal proliferation. In this study we identify several distinct roles of new polysaccharides in hMSC biology, which disclose a role of polysaccharides in hMSC proliferation. GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides in NHDF cells. But in hMSCs, GJIC was slightly inhibited in all cultured treated cells. In contrast, cell proliferation was enhanced by different polysaccharides in hMSCs (6 to 8 folds) more than in NHDF cells (2 to 5 folds) in comparison with controls. As stated earlier, in mice and monkeys, sulfated polysaccharide such as fucoidan caused increase in hematopoetic stem cells [3] and Matsubara *et al.* reported that basement membrane-like extracellular matrix (bmECM) had greater effects on the proliferation of hMSC [5]. Our result also coincided with these reports. Usually, inhibition of the function of connexin is considered to cause the cellular proliferation [4]. Therefore, these findings, that there is a relationship between the inhibitory effects on the connexin function and cellular proliferation, coincided with the result previously reported. Our studies postulated that these new polysaccharides seem to play a significant role in cell proliferation of both NHDF cells and hMSCs. Especially, these new polysaccharides are novel materials to increase the cell number of hMSCs and therefore hMSCs provide a good and clinically relevant model system. In addition, the positive effect of new polysaccharides on hMSC proliferation warrants further studies toward its exploitation in tissue engineering.

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STUDIES ON THE EFFICACY, SAFETY AND QUALITY OF THE TISSUE ENGINEERED PRODUCTS: EFFECTS OF A CATALYST USED IN THE SYNTHESIS OF BIODEGRADABLE POLYMER ON THE CHONDROGENESIS OF HUMAN ARTICULAR CARTILAGE

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Abstract: Among different synthetic biodegradable polymers, polyesters such as poly (glycolic acid) (PGA) is an attractive candidate in orthopedic applications, because of its degradation product glycolic acid is a natural metabolite. The biocompatibility of PGA that was synthesized with and without inorganic tin catalyst, in chondrogenesis of human articular cartilage (HAC) was investigated using a 4 weeks micromass culture system. PGA with tin catalyst caused significant enhancement in chondrocyte proliferation and expression of collagen type II gene. Amounts of total collagen and collagen type II protein were also increased. However, aggrecan gene expression was almost similar to control cultures. On the contrary, PGA without catalyst caused an inhibitory action on the chondrogenesis. From the viewpoint of safetyness, PGA was not suitable to use as the biodegradable scaffold for cartilage.

Key words: Human articular cartilage, Chondrogenesis, PGA, Tin catalyst.

1. INTRODUCTION

The fields of biotechnology and tissue engineering by using different synthetic biodegradable polymers are general concepts because of its disappearance in the body. In general, synthetic biodegradable polymers offer greater advantage over natural or other materials. The prime advantages include the capacity to change the mechanical properties and degradation kinetics to suit various applications. Synthetic biodegradable polymers, especially polyester

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such as poly (glycolic acid) (PGA) plays an important role in orthopedics. PGA, a polymer of glycolic acid can be synthesized under the influence of different catalysts. The common catalysts used include organotin, antimony, lead, and zinc. Organotin compounds are known agents to cause neurotoxicity [1], cytotoxicity [2], immunotoxicity and genotoxicity [3] in human and other experimental animals. Disproportionate dwarfing syndrome, affecting the limbs severely than the trunk, was observed in the rats that had been injected with certain tin compounds [4]. No study yet has reported the chondrogenic effects of PGA, synthesized with and without inorganic tin catalyst. In this study, the biocompatibility of PGA synthesized with and without tin catalyst was investigated using human articular cartilage (HAC) in a micromass culture system.

2. MATERIALS AND METHODS

2.1. ***Medium and Polymers Used for Cell Culture:*** Chondrocyte growth medium were commercially obtained from BioWhittaker, Inc., (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (Mw = 1,500) and PGA without catalyst (PGA) (Mw = 1,100) were tailor-made and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical Co. Irvine, UK).

2.2. ***Cells and Culture Methods:*** HAC of the knee joint was commercially obtained from BioWhittaker, Inc., (Walkersville, MD, USA). High-density micromass cultures were started by spotting $4x10^5$ cells in 20μl of medium onto Costar 24-well microplates for tissue culture (Costar type 3526, Corning Co. Ltd.). After 2 h of attachment period at 37°C in a CO_2 incubator, culture medium (1ml/well) was added into 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 control. The cultures were continued for 4 weeks with medium change twice in a week. At least four cultures were run for each sample.

2.3. ***Cell Proliferation Study:*** Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Ltd., Osaka, Japan) staining method. After 4 weeks culture, cells were fixed with 100% Methanol, stained by applying 0.1% crystal violet in Methanol, and washed. Again methanol was applied and the absorbance was measured at a wavelength of 590 nm using an ELISA reader (Bio-Tek Instruments, Inc.,Winooski, Vt., USA).

2.4. ***Differentiation Assay*****:** After proper washing with methanol and acetic acid, proliferation assay was followed by the differentiation assay by

staining the cells with 1% (v/v) alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 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, Inc.,Winooski, Vt., USA).

2.5. *Analytical Assays:* Commercially available assay kit [collagen assay kit, Biocolor Ltd, Newtownabbey, Northern Ireland] was used for the measurement of collagen within the cultured cells as previously described [5]. The amounts of total collagen content (acid and pepsin soluble fractions) and collagen type II protein of the cultured chondrocytes was detected as per manufacturer's instruction. The absorbance of the samples was measured at a wavelength of 540 nm using a spectrophotometer.

2.6. *Real-time polymerase chain reaction (PCR):* For detection of the presence of proteoglycans, namely 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 PCR was done using LightCycler system with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Penzberg, Germany). LightCyclerTM- Primer set (Roche Diagnostics) was used for quantitative detection of Collagen type II gene, aggrecan gene, and also a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

2.7 *Statistical Study:* Student's t test was used to compare the sample results. Statistical significance was accepted at $p < 0.05$. All values in this study are reported as means ± S.D (standard deviation).

3. RESULTS

3.1. *Cell Proliferation and Differentiation:* Cell proliferation was 1.8 (*$p < 0.05$)-fold increased in PGA (Sn) treated culture compared with DMSO group as the control. Whereas cell proliferation in PGA treated culture was almost similar to DMSO group (Figure 1A). In the case of cell differentiation, PGA (Sn) group showed a slight decrease in cell differentiation compared to DMSO control (Figure 1B).

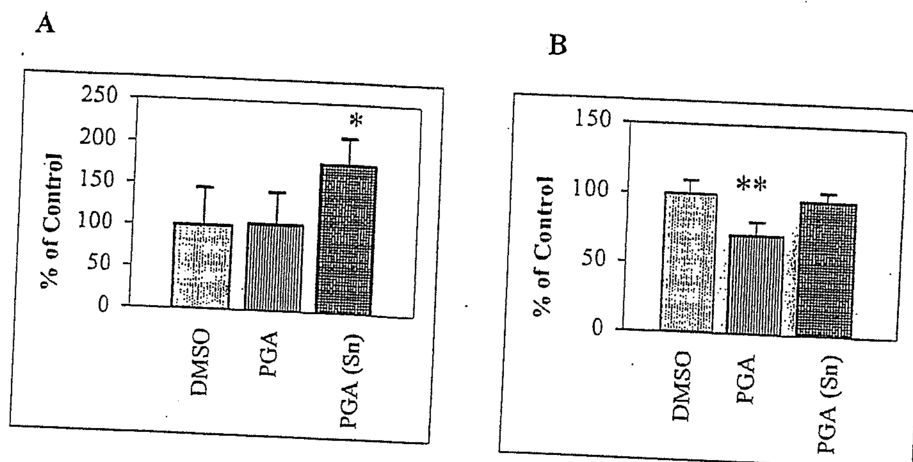

Figure 1. Cell proliferation (A) and cell differentiation (B) of human articular chondrocytes after 4 weeks culture period. * $p < 0.05$, ** $p < 0.01$.

3.2. *Extracellular matrix gene expression:* Collagen type II gene was strongly expressed in PGA (Sn) than in PGA and control group (Figure 2A). However, aggrecan gene expression was inhibited in the PGA and no difference was observed between PGA (Sn) and the control group (Figure 2B).

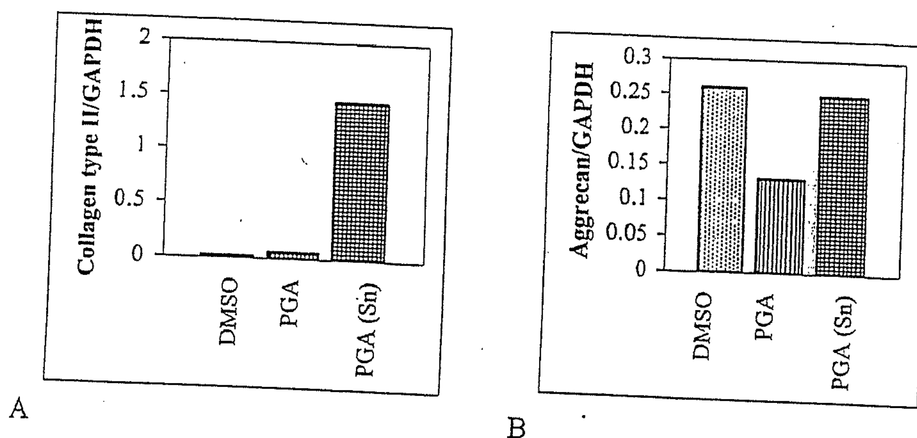

Figure 2. Expression of collagen type II gene (A) and aggrecan gene (B) in cultured chondrocytes, estimated by real time PCR method.

3.3. *Measurement of Collagen type II protein and Total collagen amount:* The amount of pepsin soluble and cartilage specific protein, collagen type II was significantly increased (**$p < 0.01$) in PGA (Sn) group, but almost no difference in the amount was observed between the PGA and control group (Figure 3A). The amount of total collagen (both acid and pepsin soluble protein) was significantly increased (**$p < 0.01$) in PGA (Sn) group compared with the controls. (Figure 3B).

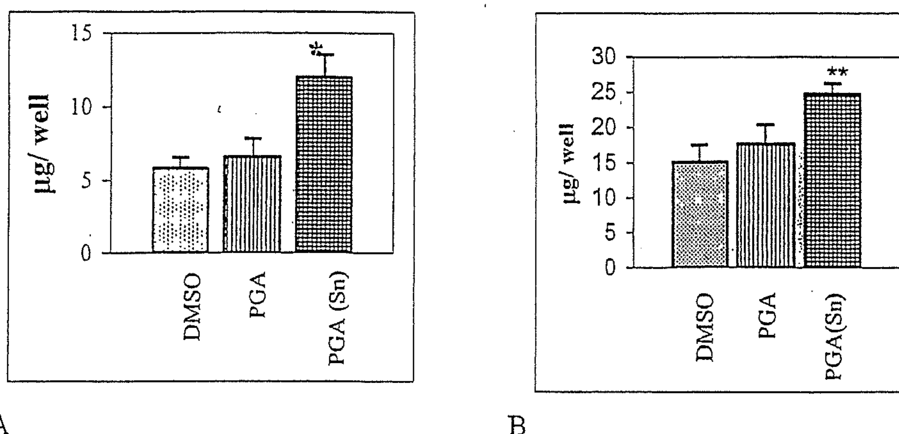

Figure 3. Estimation of the amount of collagen type II protein (A) and total collagen (B) of human articular chondrocytes after 4 weeks of culture. ** $p < 0.01$.

4. DISCUSSION

Different tin compounds had already exhibited general cytotoxic effects on rabbit articular cartilage in monolayer culture [6], and Yamaguchi et al. suggested bone as the critical organ in inorganic tin toxicity in rats [7]. We evaluated the chondrogenic effect of HAC with PGA, synthesized with and without inorganic tin catalyst, in micromass culture system. Oral administration of certain tin compounds was reported to exert stimulatory effect on chondrocyte proliferation of rat [6]. Parallel with this event, proliferation assay of HAC with PGA (Sn) performed in our study also showed stimulatory effect on chondrocyte proliferation in micromass culture (Fig 1). But, PGA showed neither inhibition nor stimulation on the chondrocyte proliferation and thus inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In rat, oral administration of inorganic tin was reported to cause decrease in the proliferation of the chondrocytes accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis [8]. These references suggested a direct relation of inorganic tin in chondrocyte proliferation with the synthesis of collagen protein. In support of these suggestions, our results also showed enhancement of HAC proliferation, expression of collagen type II gene, and amounts of total collagen and collagen type II protein. There was a strong decrease in aggrecan gene expression in PGA compared with control. This study firstly to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. We speculate that nature of tin compound, and also the route of application may play a key role in exhibiting various chondrogenic effects of this metallic compound. In

spite of different positive findings regarding human articular chondrogenesis, from the view points of safetyness we are considering inorganic tin catalyst is not appropriate to use for synthesis of biodegradable polymers in future clinical applications.

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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.

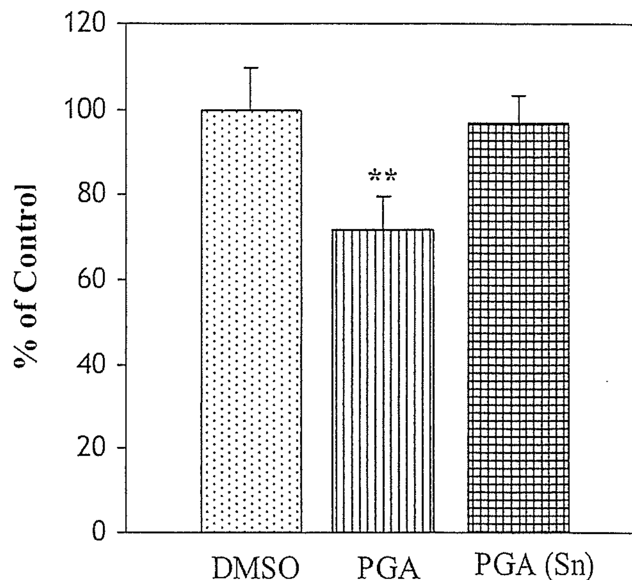

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

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.

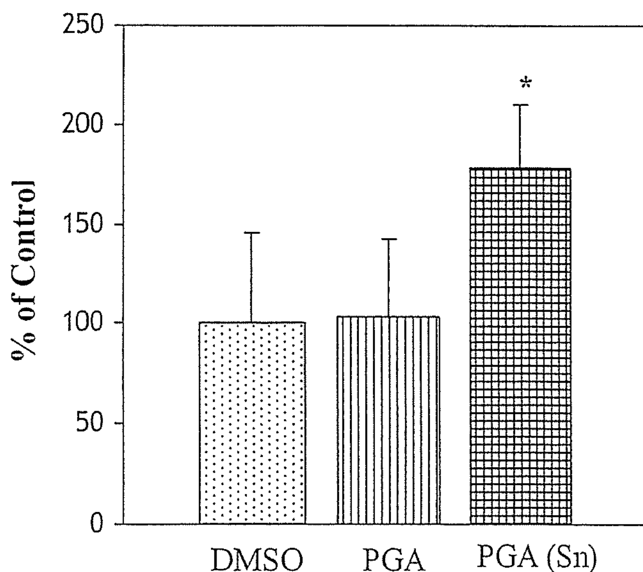

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

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

Extracellular matrix gene expression

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

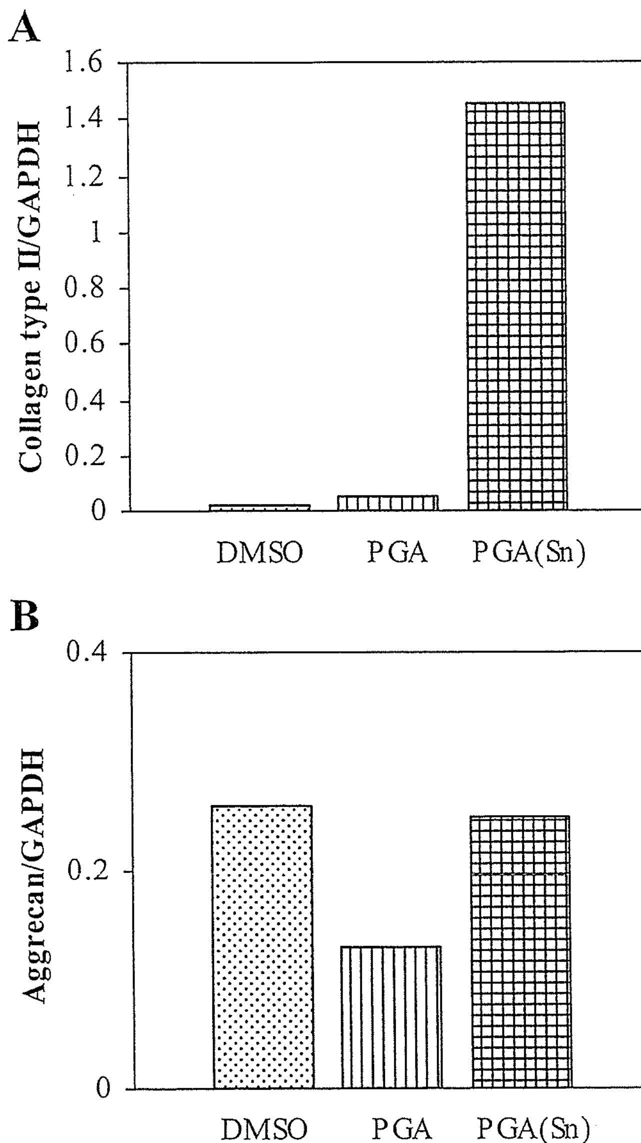

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between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

Measurement of collagen type II protein

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

Measurement of total collagen

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

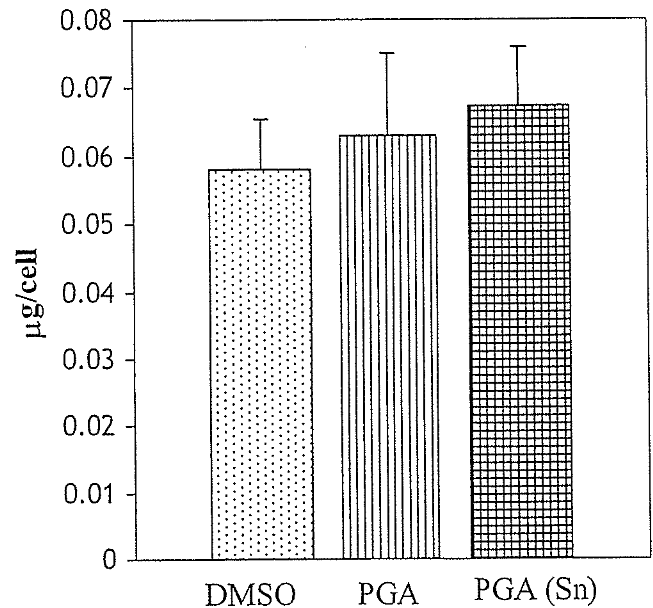

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

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

Estimation of sulfated glycosaminoglycan concentration

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

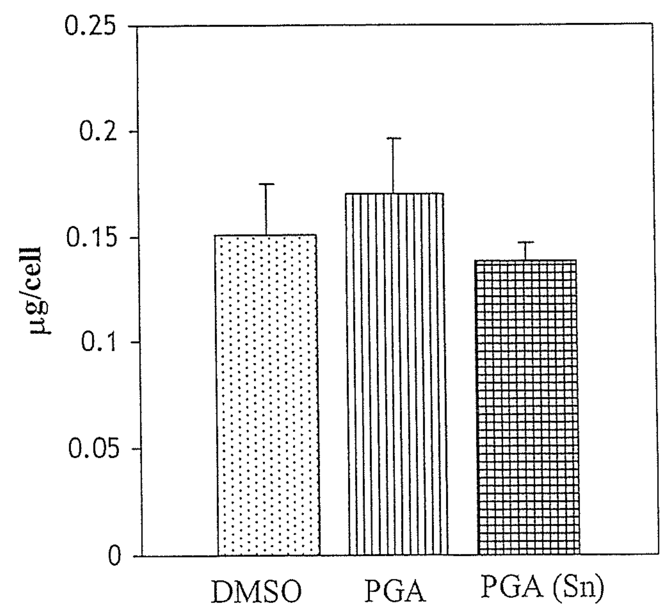

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

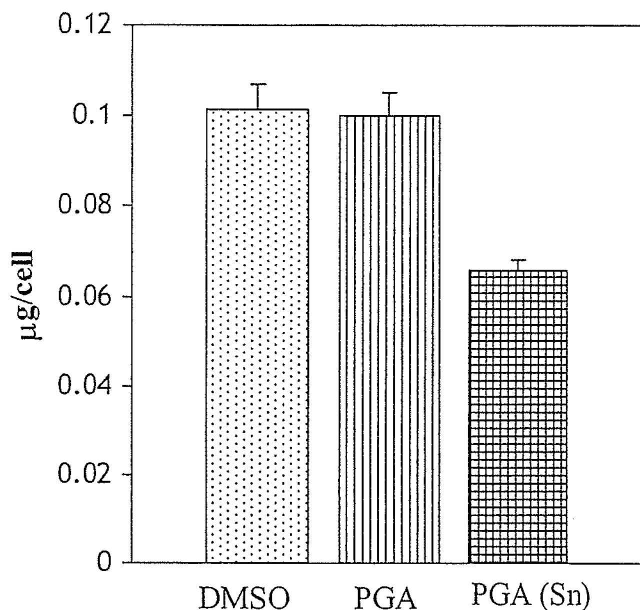

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

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

DISCUSSION

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

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

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

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

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