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A mouse strain difference in tumorigenesis induced by biodegradable polymers

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Abstract: The use of poly-L-lactic acid (PLLA) surgical implants for repair of bone fractures has gained popularity in the past decade. The aim of this study was to evaluate the *in vivo* effect of PLLA plates on subcutaneous tissue in two mouse strains, BALB/cJ and SJL/J, which have higher and lower tumorigenicity, respectively. Gap-junctional intercellular communication and protein expression of connexin 43 were significantly suppressed, whereas secretion of transforming growth factor-β1 and expression of extracellular matrix, insulin-like growth factor binding protein 3, and cysteine-rich intestinal protein 2 were significantly increased in PLLA-implanted BALB/cJ mice when compared with BALB/cJ controls. Finally, tumors were formed after implantation of cultured cells from the more-tumorigenic BALB/cJ, but not SJL/J, mice into nude mice. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 409–417, 2006

Key words: poly-L-lactic acid; gap-junctional intercellular communication; transforming growth factor-β1; connexin 43; nude mice

INTRODUCTION

The morphologic, chemical, and surface electrical characteristics of a biomaterial can influence the extent of the cellular response to an implant,[1,2] but host factors also contribute, so that an identical material implanted in different species[3,4] or at different anatomical locations[5,6] may elicit different degrees of response. Poly-L-lactic acid (PLLA) is a synthetic degradable polymer with good biocompatibility that is widely used clinically for surgical implants and as a bioabsorbable suture material.[7,8] Long-term implants of PLLA produced tumors in rats,[9] and adverse effects were also reported in other animal experiments.[10] All tumors are generally viewed as the result of disruption of the homeostatic regulation of the cell's ability to respond to extracellular signals, which triggers intracellular signal transduction abnormalities.[11] During the transition from the single-cell organism to the multicellular organism, many genes evolved to regulate these cellular functions. One of these genes is the gene coding for a membrane-associated protein channel (the gap junction).[12] Gap-junctional intercellular communication (GJIC) involves two hemichannels or connexons,[13] and each connexon is composed of six basic protein subunits named connexin (Cx), which allow the cell–cell transfer of small molecules. Approximately 20 connexins are known, and they are expressed in a cell- and development-specific manner.[14,15] GJIC also plays an important role in the maintenance of cell homeostasis and in the control of cell growth.[16] Thus, disruption of GJIC has been shown to contribute to the multi-step, multi-mechanism process of carcinogenesis.[17–19] Several tumor-promoting agents have been shown to restrict GJIC by phosphorylation of connexin proteins, such as connexin 43, which is essential in forming the gap junction channel.[20,21] Our previous study revealed that PLLA increased the secretion of transforming growth factor-β1 (TGF-β1), suppressed the mRNA expression of Cx 43, and inhibited GJIC in the early stage after implantation, thus promoting tumorigenesis in BALB/cJ mice.[22] We have hypothesized that the difference in tumorigenic potentials of PLLA is caused mainly by the different tumor-promoting activities of these biomaterials and that TGF-β1 might have an important role in PLLA-implanted BALB/cJ mice. Therefore, in our present experimental approach, we aimed to determine the novel effects of PLLA plates in two mouse strains, BALB/cJ and SJL/J, after long-term implantation. Among mouse strains, the former is a more tumorigenic strain when compared with the later.[23]

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Immune-deficient nude mice, which are highly susceptible to tumorigenicity, were also used in this experiment.

MATERIALS AND METHODS

Animals

Five-week-old female BALB/cJ and SJL/J, and five-week-old male BALB/cAnCrj-nu mice were purchased from Charles River (Japan) and maintained in the animal center according to the NIHS animal welfare guidelines. All mice were fed standard pellet diets and water *ad libitum* before and after PLLA implantation.

Implantation of PLLA

PLLA was obtained from Shimadzu Co. Ltd. as uniform sheets. The implants (size, 20 × 10 × 1 mm^3; Mw, 200,000) were sterilized using ethylene oxide gas prior to use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. The dorsal skin was shaved and scrubbed with 70% alcohol. Using an aseptic technique, an incision of about 2 cm was made; a subcutaneous pocket was formed by blunt dissection away from the incision, and one piece of PLLA was placed in the pocket. The incision was closed with silk sutures. In both strains, controls were obtained by sham operation and subsequent subcutaneous pocket formation. Following surgery, the mice were housed in individual cages. After 10 months, mice from the implanted group were killed, implanted materials were excised, and subcutaneous tissues from the adjacent sites were collected for culture. At the same time, subcutaneous tissues were removed from the sites in the sham-operated controls that correlated with the implant sites. Similar experiments were also performed 1 month after PLLA implantation.[22]

Cell culture of subcutaneous tissues

The subcutaneous tissues were maintained in minimum essential medium (MEM) supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C.

Giemsa staining

When cells reached confluence in tissue culture dishes, they were fixed and stained with Giemsa solution. Cell morphology was determined under an inverted light microscope.

Western blot analysis

When cells had grown confluent in 60-mm tissue culture dishes, all cells were lysed directly in 100 μL 2% sodium dodecyl sulfate (SDS) gel loading buffer (50 m*M* Tris-HCl, pH 6.8, 100 m*M* 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). The protein concentration of the cleared lysate was measured using a micro-plate BCA protein assay (Pierce, Rockford, IL). Equivalent protein samples were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK, Buckinghamshire, UK), and Cx 43 protein was detected by anti-Cx 43 polyclonal antibodies (ZYMED Laboratories, San Francisco, CA). The membrane was soaked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan), reacted with the anti-Cx 43 polyclonal antibodies for 1 h, and after washes with phosphate-buffered saline (PBS) containing 0.1% Tween20, reacted with the secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase for 1 h. After several washes with PBS-Tween20, the membrane was detected with the ECL detection system (Amersham Pharmacia Biotech UK).

Scrape-loading and dye transfer assay

The scrape-loading and dye transfer (SLDT) technique was performed by the method of El-Fouly et al.[24] Confluent monolayer cells in 35-mm culture dishes were used. After rinsing with Ca^{2+}, Mg^{2+} PBS(+), cell dishes were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR) in PBS(+) solution and were 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 transfer was monitored using a fluorescence microscope equipped with a type UFX-DXII CCD camera and a super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay

Cells were seeded onto 60-mm dishes. The conditioned medium was collected after centrifugation at 1000 rpm for 2 min. The TGF-β1 levels of the media were measured with commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

DNA microarray analysis

At least 10^7 cells were harvested and frozen in liquid nitrogen. Total RNA was extracted, purified, and assessed for yield and purity, and cDNA probes were synthesized with the Atlas™ Pure Total RNA Labeling System (Clontech) according to the manufacturer's instructions. Hybridization of the ^{33}P-labeled probes to the Atlas Array of Mouse Cancer 1.2 k Array (Clontec 7858–1), on which 1176 cDNAs

of cancer-related genes were spotted, was performed with Atlas™ cDNA Expression Arrays according to the manufacturer's instructions. The phosphor images of hybridized arrays were analyzed with AtlasImage™ (Clontech). Genes that were up- or downregulated more than fivefold relative to the negative controls are discussed.

Determination of tumorigenicity in nude mice

Cultured cells were harvested by trypsinization, and 2×10^6 washed cells suspended in 0.2 mL of PBS were inoculated at a single subcutaneous site into 6–8-week-old nude mice. All mice were examined regularly for the development of tumor.

Soft agar assay

Approximately 100,000 cells per well from each clone were seeded in 2 mL of 0.3% soft agar in culture medium on a solidified basal layer in 6-well tissue culture plates. The plates were cultured for 4 weeks and then stained with *p*-iodotetrazolium violet for 48 h before counting.

Statistical analysis

Student *t* tests were used to assess whether differences observed between the implanted and control samples were statically 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. The confidence level was set at 95% for all tests. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD.

RESULTS

Giemsa staining

Cells with different morphologies formed a slightly crisscrossed pattern in the BALB/cJ control group, whereas cells in the implanted groups of BALB/cJ showed a markedly crisscrossed pattern. The cells were extensively piled up, which decreased contact inhibition, under inverted light microscopy observation and Giemsa staining [Fig. 1(A,B)]. In contrast, the cells of the SJL/J group formed a parallel, flat, confluent monolayer that maintained contact inhibition [Fig. 1(C,D)].

Western blot analysis

We examined the protein expression of the connexin 43 gene and found that the total protein level was significantly decreased in PLLA-implanted BALB/cJ mice when compared with that in BALB/cJ controls (Fig. 2). However, protein expression was decreased in both control and PLLA-implanted groups in SJL/J mice (Fig. 2).

SLDT assay

The SLDT assay was used to assess functional GJIC. GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice when compared with that in BALB/cJ controls (Fig. 3). A significant difference was also observed between the two strains of mice in that the GJIC was lower in SJL/J than in BALB/cJ group (Fig. 3).

ELISA

The secretion of TGF-β1 was significantly increased in PLLA-implanted BALB/cJ subcutaneous tissues in comparison with that from BALB/cJ control mice. On the contrary, secretion of TGF-β1 tended to decrease in the SJL/J implanted mice when compared with that in SJL/J control mice (Fig. 4).

DNA microarray analysis of the four kinds of cells

Expression of the major ECM [fibronectin 1, procollagen VIIIα 1, and osteopontin precursor (OPN)] proteins [Fig. 5(A–C)], insulin-like growth factor binding protein (IGFBP) 3 [Fig. 5(D)], and cysteine-rich intestinal protein 2 (CRIP 2) [Fig. 5(E)] were increased in the PLLA-implanted BALB/cJ mouse cells when compared with that in BALB/cJ control mouse cells. No such difference was observed between SJL/J implanted and control mouse cells.

Tumorigenicity in nude mice

No tumor was formed in PBS(−) injected nude mice [Fig. 6(A)]. Rapid growth of large tumors was observed in nude mice within 2 weeks of injection of cultured cells from PLLA-implanted BALB/cJ mice [Fig. 6(B,C,E,F)]. Nude mice injected with HeLa cells, which served as positive controls, showed slower growth of tumor 4 weeks after cell injection [Fig. 6(D,G)].

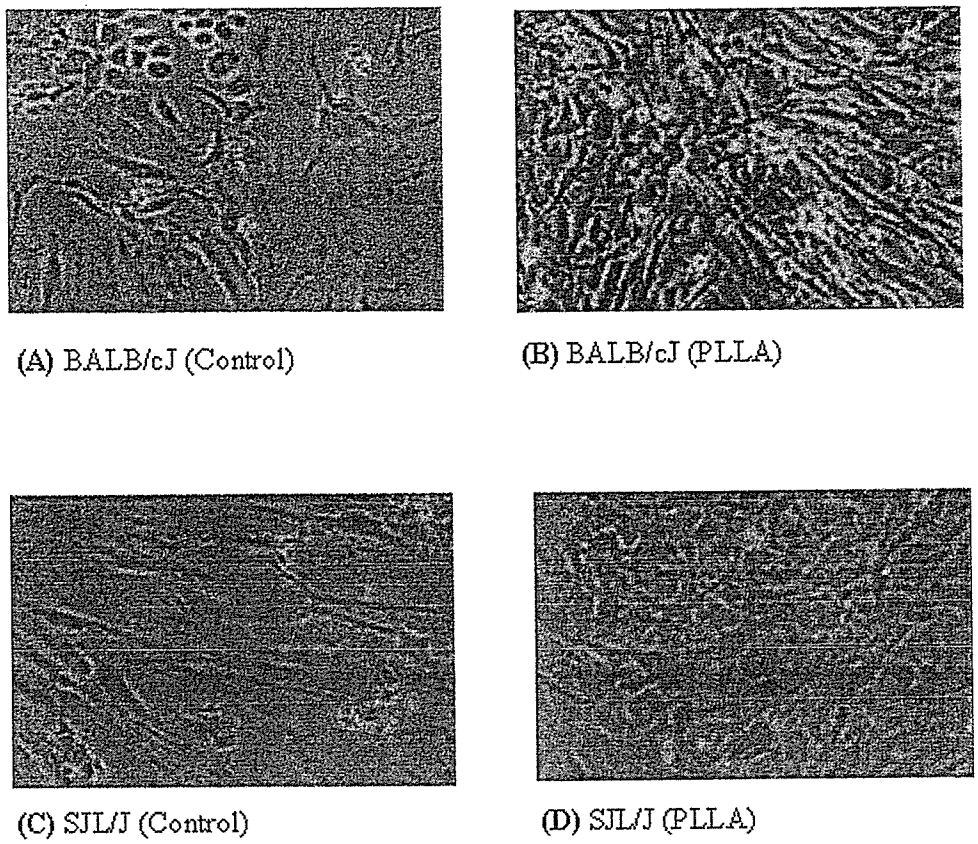

Figure 1. Mouse cell morphology. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Inverted light microscopic appearance (magnification ×100) of (A) BALB/cJ (control), (B) BALB/cJ (PLLA), (C) SJL/J (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

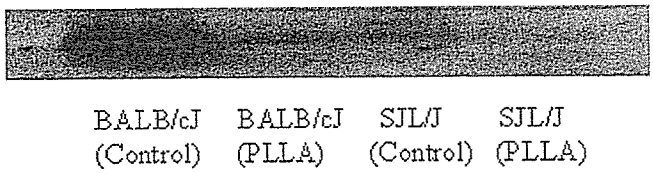

Figure 2. Expression of Cx 43 protein by Western blot analysis. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Total protein expression was significantly decreased in PLLA-implanted BALB/cJ mice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

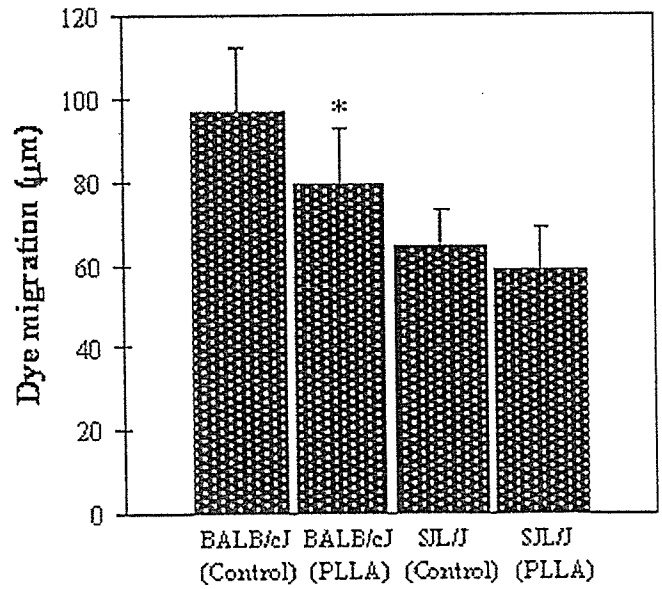

Figure 3. Statistical analysis of SLDT assay. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. GJIC was found to be significantly inhibited in PLLA-implanted BALB/cJ mice cells when compared with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

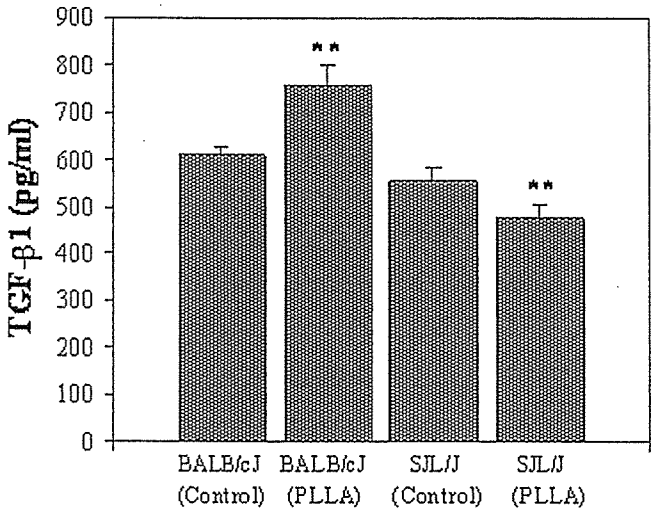

Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. Three each of both implanted mice and sham-operated controls were killed after 10 months. Results shown are representative of two independent experiments. Secretion of TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ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

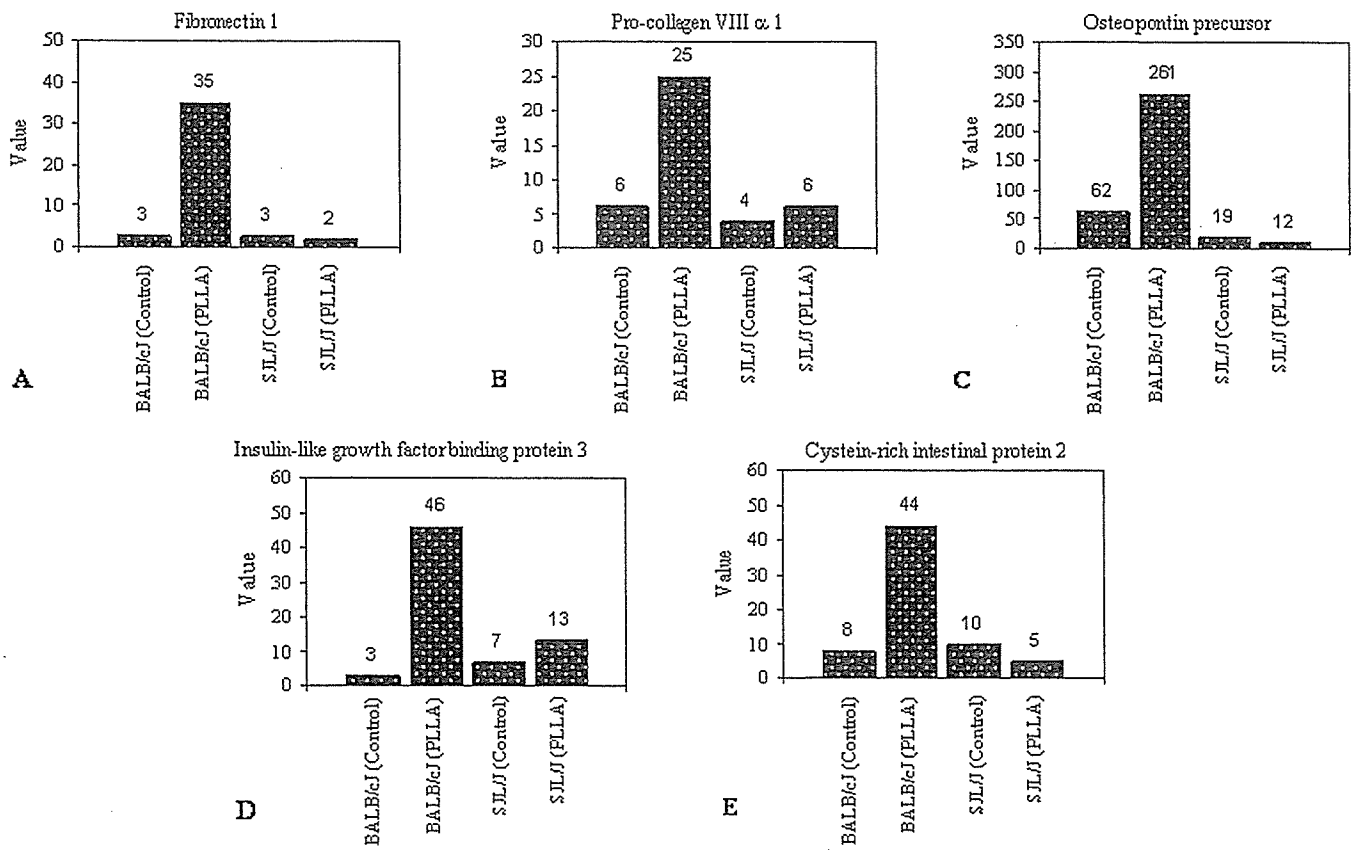

Figure 5. DNA microarray analysis of these four kinds of cells. The expression of (A) fibronectin 1, (B) pro-collagen VIIIα 1, (C) osteopontin precursor (OPN), (D) insulin-like growth factor binding protein (IGFBP) 3, and (E) cysteine-rich intestinal protein 2 (CRIP 2) increased in the cells of PLLA-implanted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

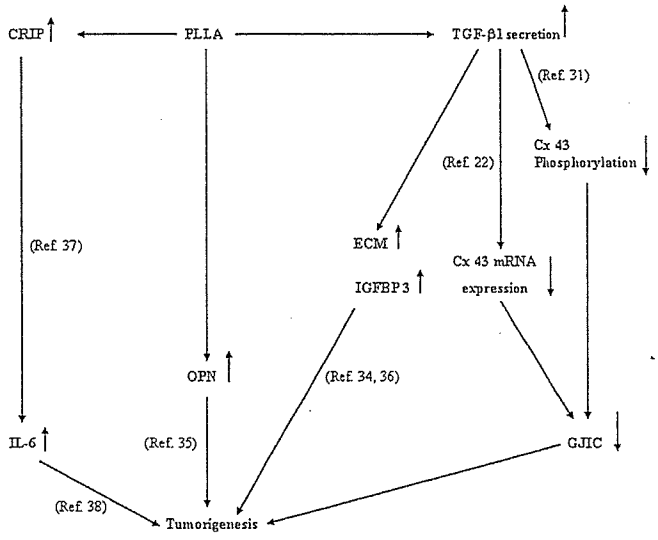

Figure 8. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

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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

S. Iijima and K.-I. Nishijima (eds.), Animal Cell Technology: Basic & Applied Aspects, 81–85.

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).

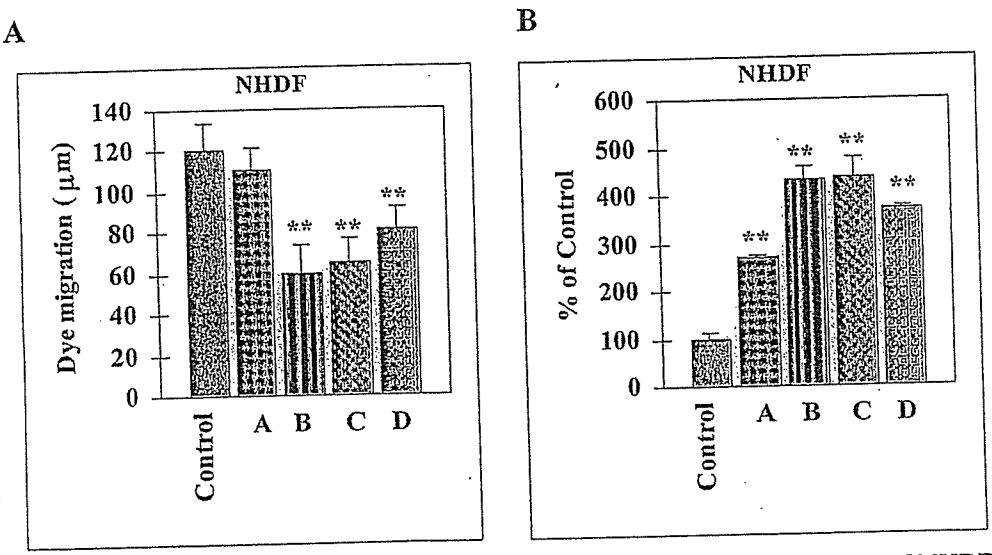

Figure 1. In A, Statistical analysis of SLDT assay and in B, cell proliferation of NHDF cells. ** p < 0.01.

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.

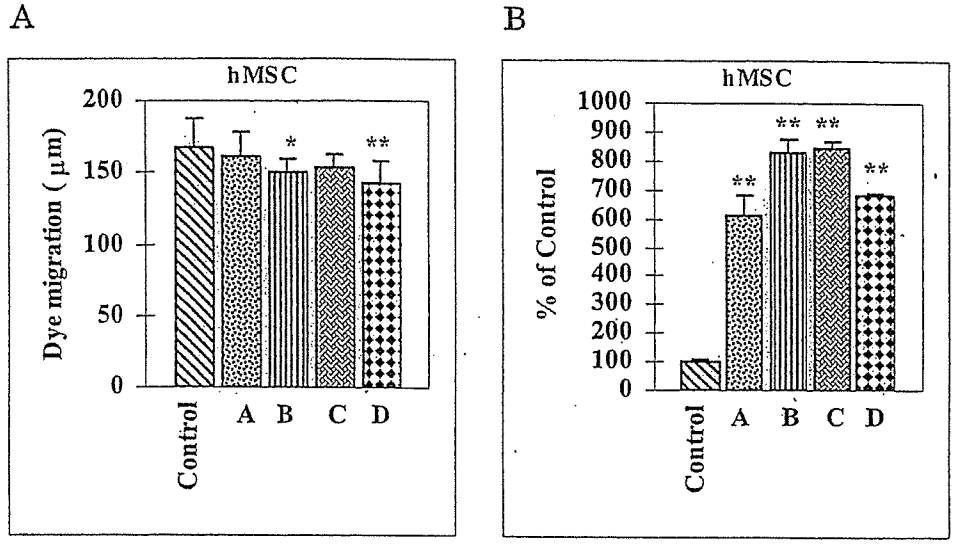

Figure 2. In A, Statistical analysis of SLDT assay and in B, cell proliferation of hMSCs. * $p < 0.05$, ** $p < 0.01$.

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).

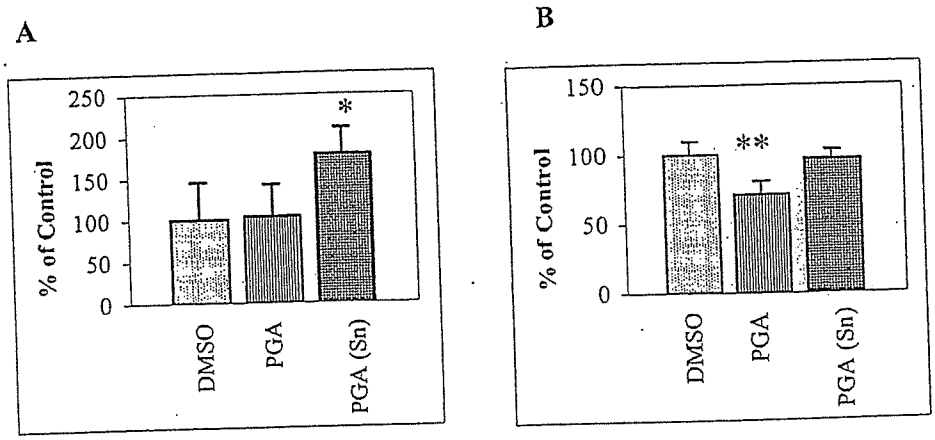


Figure 1. Cell proliferation (A) and cell differentiation (B) of human articular chondrocytes after 4 weeks culture period. * $p < 0.05$, ** $p < 0.01$.

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).

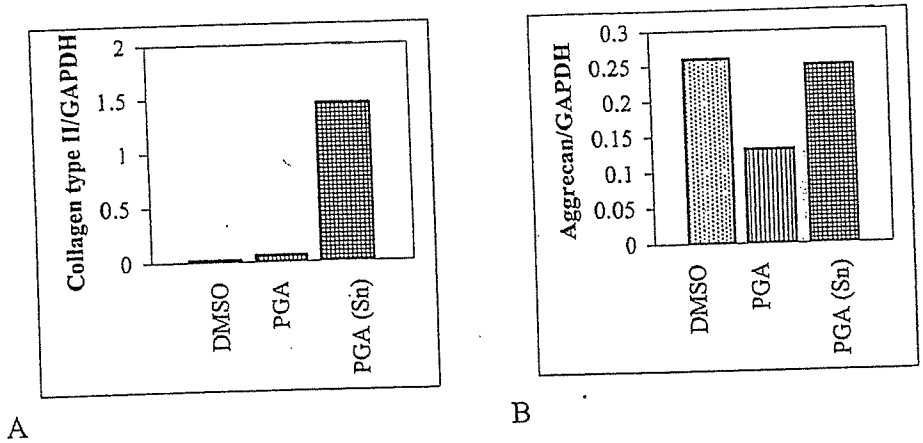
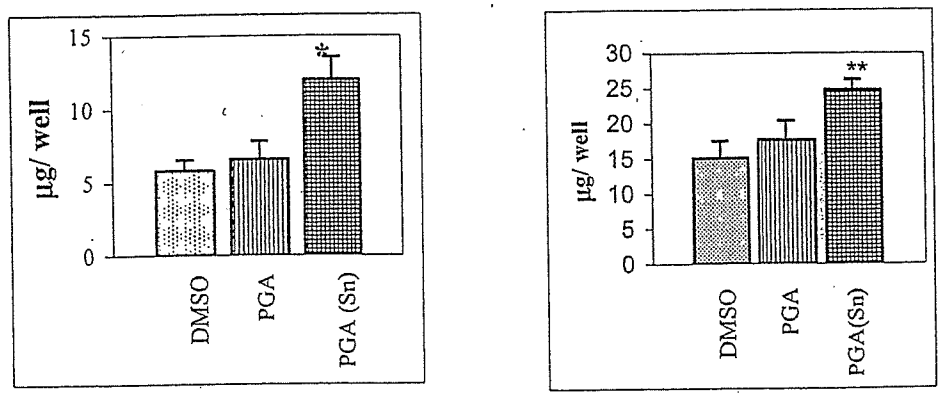


Figure 2. Expression of collagen type II gene (A) and aggrecan gene (B) in cultured chondrocytes, estimated by real time PCR method.

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).



A B

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