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).

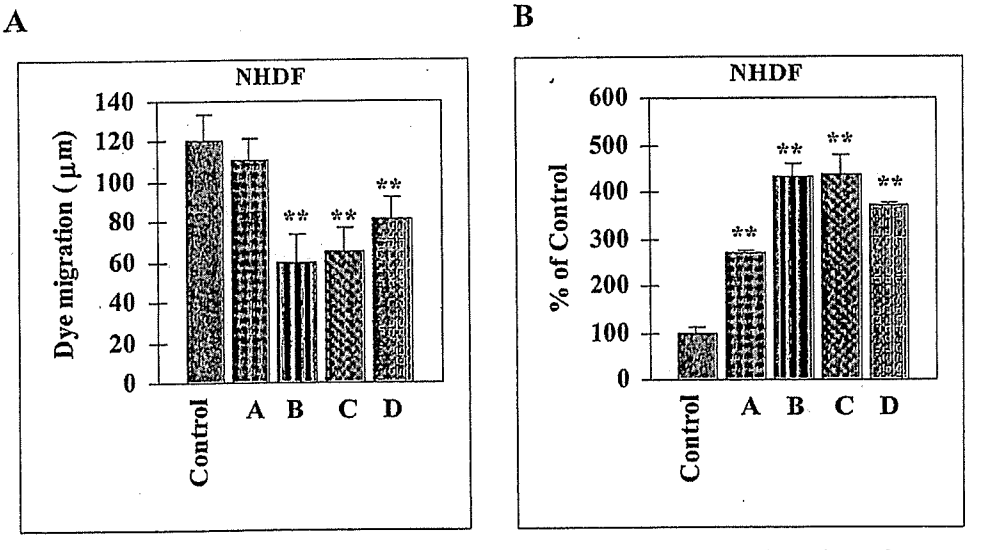

Figure 1. In A, Statistical analysis of SLDT assay and in B, cell proliferation of NHDF cells. ** p < 0.01.

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.

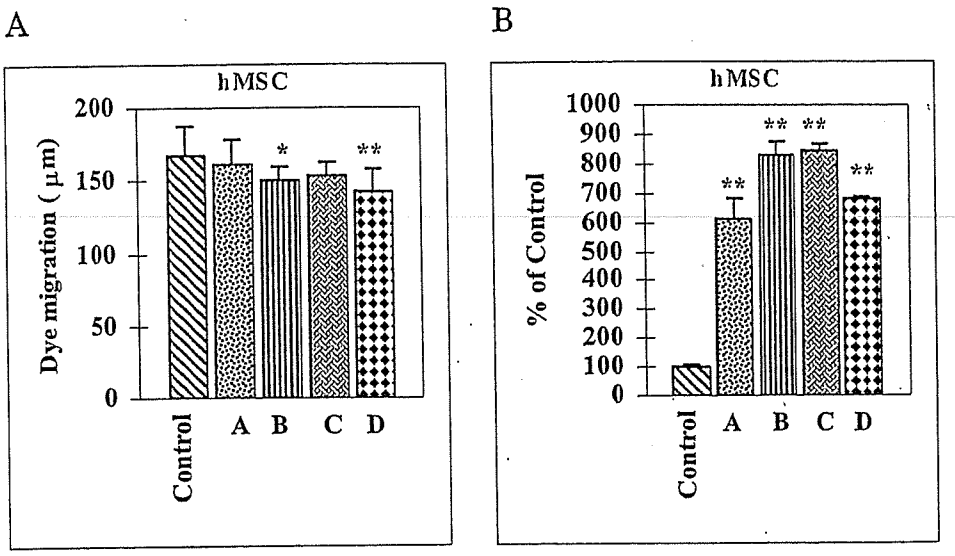

Figure 2. In A, Statistical analysis of SLDT assay and in B, cell proliferation of hMSCs. * $p < 0.05$, ** $p < 0.01$.

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).

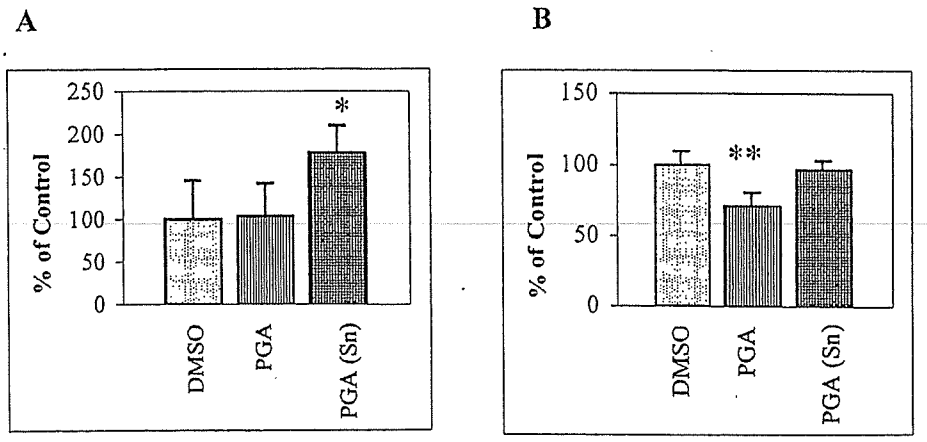


Figure 1. Cell proliferation (A) and cell differentiation (B) of human articular chondrocytes after 4 weeks culture period. * $p < 0.05$, ** $p < 0.01$.

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).

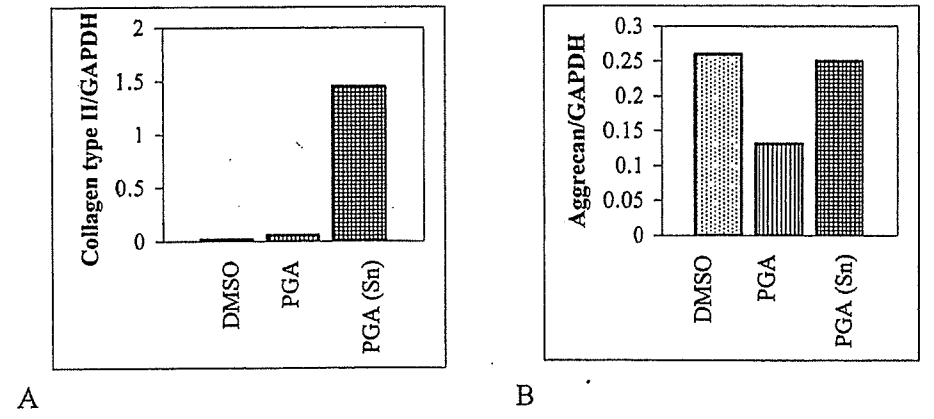
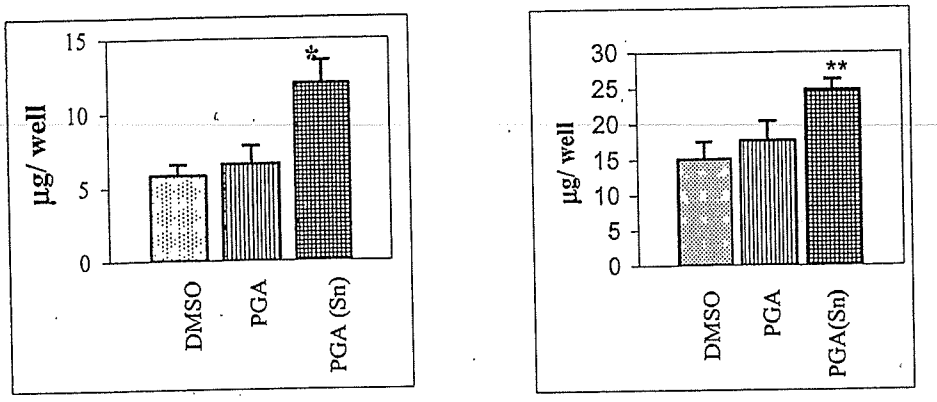


Figure 2. Expression of collagen type II gene (A) and aggrecan gene (B) in cultured chondrocytes, estimated by real time PCR method.

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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INCREASE IN THE INSULIN SECRETION OF HIT-T15 CELLS:

Gap Junctional Intercellular Communications Enhanced by Hyaluronic Acid

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Abstract: Gap junctional intracellular communications (GJIC) were found in almost all types of vertebrate cells. The β-cells of the endocrine pancreas are connected by gap junctions, and the membrane specializations are thought to provide channels for direct cell-to-cell and cell-to-matrix communications. Previous studies suggested that GJIC may participate in the control of insulin secretion. It has been suggested that hyaluronic acid (HA) increases the function of GJIC—*via* the expression of Connexin43, a major protein component of gap junctions. However, the effects of HA on insulin secretion and gap-junctions between β-cells remains unclear. To determine whether insulin secretion is affected by gap-junctions after HA-treatment, we exposed HIT-T15, a clonal pancreatic β-cell line, in various concentrations of HA for 72 h, and detected their base- and glucose-stimulated insulin secretion, using an insulin assay kit by ELISA technique. The cellular functions of GJIC were assayed by dye transfer method using the dye solution of Lucifer Yellow. HA-treatment resulted in the enhancement of GJIC and the increase of insulin release. The results obtained in this study suggest that HA increases the insulin secretion of HIT-T15 cells by the enhancement of GJIC.

Key words: hyaluronic acid; gap junction; HIT-T15 cells; insulin secretion.

1. INTRODUCTION

Gap junctions are channels between cells for the passage of ions, small metabolites, and second messengers. The physical link is responsible for electrical and metabolic communications in several types of cells, including the insulin-producing pancreatic β-cells. The insulin secretion from pancreatic β-cells is a multicellular event arising as an emergent property due to β-cell intercellular communications. Among

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the several mechanisms to control cell-to-cell communications between pancreatic β-cells, the one mediated by gap junctions is believed to be essential for the recruitment and synchronization of insulin-secreting cells. Previous studies showed that the proper insulin secretion from pancreatic islets depends on a communication network coordinating the activities of individual insulin-producing cells. The single β-cells unconnected with connexin channels show poor expression of the insulin gene and release low amounts of the hormone after stimulation, whereas both insulin biosynthesis and release are rapidly improved due to the restoration of β-cell contacts [1, 2]. It is known that HA-treatment enhances the function of GJIC in normal human dermal fibroblasts [3] and the expression of Connexin43 in rat calvarial osteoblast [4]. In this study, we used HIT-T15 cells, the clonal pancreatic β-cell line, to observe the relative effect of HA on insulin secretion and gap-junctions between β-cells. The results obtained indicate that HA increases insulin secretion of HIT-T15 cells by the enhancement of GJIC.

2. MATERIALS AND METHODS

2.1 Preparation of media and culture dishes

The high-molecular-weight (HMW) HA polysaccharide was dissolved in distilled water at a concentration of 4 mg/ml. Each 35-mm culture dish was coated at a final concentration of 0.01 to 2.0 mg/ml. The HA-coated dishes were dried further under sterile air flow at room temperature for 12 h before use. In order to investigate the effect of HA-addition on the functions of HIT-T15 cells, many media were prepared with various concentrations of HA.

2.2 Cell culture

The hamster pancreatic β-cell line, HIT-T15, were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 100 IU/ml penicillin-G and 100 μg/ml streptomycin. HIT-T15 cells in RPMI 1640 medium were maintained in a humidified 5% CO_2 incubator at 37°C. The subcultured cells were seeded at a density of 1.0~5.0 $\times 10^5$ cells/ml in multiwell plates or culture dishes. When they reached more than 80% confluence, the cells were used for various studies. Throughout the cell growth period the culture media were exchanged every 2-3 days.

2.3 Measurement of cell viability

HIT-T15 cells (1×10^5) were incubated into the various concentrations of HA-coated 24 wells plate, or after the cells were seeded onto 24 well plates and pre-incubated in a 10%FBS/RPMI 1640 medium overnight, the medium was exchanged for 10%FBS/HA/RPMI 1640 medium prepared. After 72 h of HA-treatment, the cell viability was determined by alamarBlue™ assay, according to the manufacturer instructions. Control cells received fresh medium without HA.

2.4 Measurement of insulin release

HIT-T15 cells were treated as described above. After washing with KRB buffer, the cells were incubated with KRB buffer for 60 min. The amount of insulin release in the spent medium was determined by ELISA insulin kit, according to the manufacturer instructions.

2.5 Scrape-loading and dye transfer (SLDT) assay

HIT-T15 cells (5×10^5) were treated as described above. The cells were washed three times with PBS (+) before the addition of the fluorescent dye. The cells were scraped using a surgical blade and loaded with 0.1% Lucifer Yellow solution for 5 min at 37°C. The dye solution was discarded, washed three times with PBS (+) solution to remove detached cells and background fluorescence. The distance of dye transfer was measured at room temperature under the fluorescence microscope equipped with a type UFX-DXII and Super High Pressure Mercury Lamp Power Supply (NIKON, Japan).

3. RESULTS AND DISCUSSION

In order to evaluate the effect of HA on cell viability, HIT-T15 cells were treated with HA-coated or -added for 72 h. At the same incubated time, the cell viability of HIT-T15 cells grown on high concentration HA-coated dishes (≧ 2.0 mg/dish) was significantly less than low concentration HA-coated and control (Fig. 1). However, there was no difference in cell viability between the HA-added and control (data not shown). Previous studies have shown that HMW (310 kDa and 800 kDa) HA-coating resulted in low adhesiveness to the cells. Because the HMW HA-coated surface provides a stable anionic surface that prevents cells attachment at the early time. In this study, after 12 h, the cells in low

concentration HA (1680 kDa)-coated dishes (0.01, 0.5, 1.0 mg/dish) already had attached and confluent but not in high concentration HA-coated dishes (2.0 mg/dish). These results indicated that the changes of cell viability by HA-treatment may depend on the cell attachment activity.

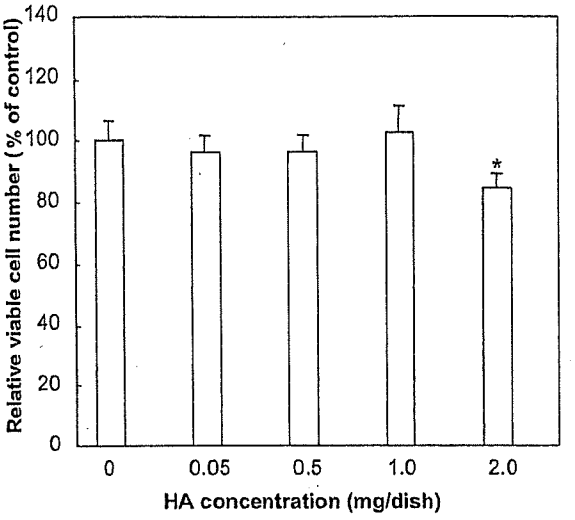

Figure 1. Viability of HIT-T15 cell after 72 h of HA-treatment. The viable cell numbers of HIT-T15 cell were determined by alamarBlue™ assay as described in Section 2. Each value denotes the mean ± S.D. $^{*}P \leq 0.05$ compared to untreated control.

HIT-T15 cells, retain glucose-stimulated insulin secretion, showed a increase in insulin secretion as a function of stimulation. Thus, their insulin output was 13.25 ± 0.96 and 19.63 ± 0.98 pg/μg protein in the base and glucose-stimulation (11.1 mM), respectively (n = 9 dishes from three independent experiments) (data not shown). When these cells were exposed to low concentration of HA-coating (0.25, 0.5. 1.0 mg/dish), their insulin secretion was significantly increased in the absence or presence of glucose-stimulation. By contrast, high concentration of HA-coating (2.0 mg/dish) failed to increase its insulin secretion (Fig. 2).

On the other hand, when HIT-T15 cells were treated with HA-addition for 72 h, the increasing effect was not exhibited. The insulin secretion was without difference between control and HA-addition (data not shown). Previous studies have indicated that HA-treatment enhances the function of GJIC in normal human dermal fibroblasts [3] and the expression of Connexin43 in rat calvarial osteoblast [4]. The increasing evidence suggests that gap junction proteins and/or GJIC participate in the multifactorial control of insulin secretion. Thus, the increase in insulin secretion by HA-coating might have relation to gap junctions.

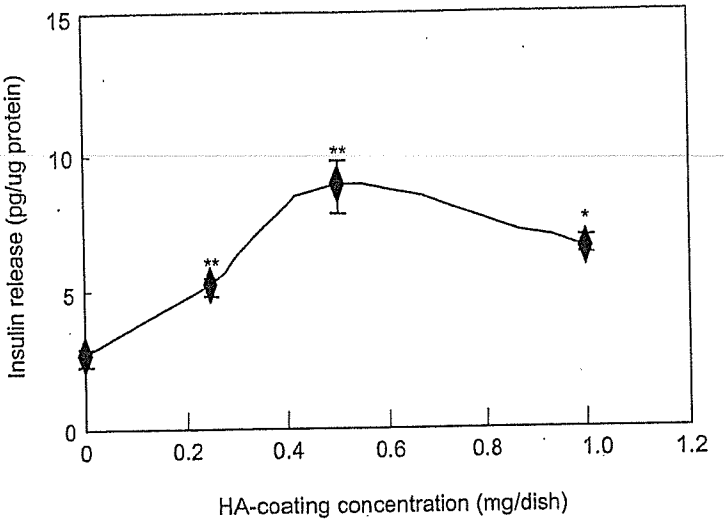

Figure 2. Concentration-dependent effects of HA-coating on insulin secretion from HIT-T15 cells. Treated with HA for 72 h, HIT-T15 cells were incubated with KRB buffer for 60 min. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean ± S.D. of three separate experiments. $*P \leq 0.05$, $**P \leq 0.01$ compared to control.

To test whether the HA-coating affects the gap junctions in pancreatic β-cells, we assessed the function of GJIC using Lucifer Yellow by SLDT assay. A scrape line was made on the cell grown to confluence, and the fluorescent dye penetrated the adjacent cells. The distance of dye transfer was determined.

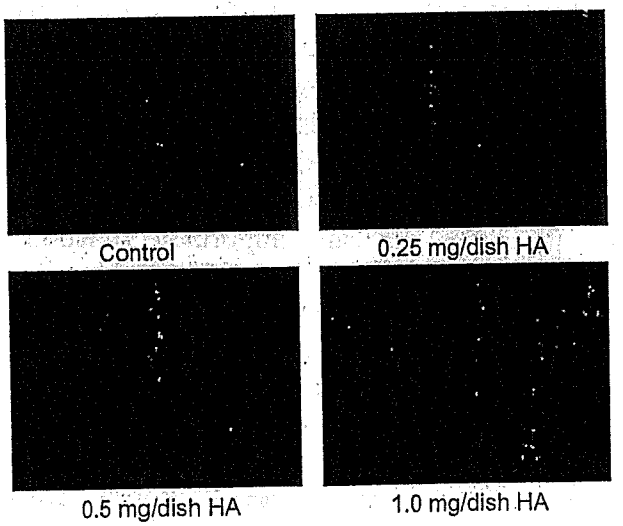

Figure 3. Time-course effects of various concentrations of HA-coating on the dye transfer ratio of HIT-T15 cells.

Fig. 3 shows the patterns of dye transfer in HIT-T15 cells treated with HA. The dye transfer extent of the cells grown on the HA-coated dishes

was more than that of the control, which indicated that GJIC function was activated by the HA-coating. The effect of HA is influenced by the concentration of FBS and the nutrients in medium, because the serum contains many components such as hormone, growth factor (FGF, etc.), cell adhesion molecule (N-CAM and cadherins), and transportation protein [4]. As a result, the HIT-T15 cells can use these nutrients and the nutrient-enriched substrata, e.g., natural extracellular matrixes with HA bound, to change the cell aggregations. Therefore, HA might play an important role in the increase of GJIC.

With the evidence above, it is known that the gap junction channels play a role in the regulation of β-cell secretion [5, 6]. It has been shown that the increase in connexin, e.g., gap junction proteins Cx43, affects the electrical coupling, synchronization of $[Ca^{2+}]_i$ oscillations, and insulin secretion, and the insulin secretion is evoked by a variety of metabolizable and nonmetabolizable secretagogues that activate different intracellular pathways [7-10]. In this study, we have found that the functional gap junction is promoted by low concentration HA-coating in spite of the inhibitory effects on the cell viability in high concentration HA-coating dishes. However, further intensive investigation should be promoted on the detailed action mechanism of HMW-HA responsible for the insulin-secreting activity.

4. CONCLUSIONS

The function of GJIC is considered to be a useful marker for evaluating tissue-engineered products. The data obtained in this study shows that gap junctions contribute to regulating some still-unknown mechanism to couple the stimulus-secretion of HIT-T15 cells under the condition of low concentration HA-coating. These results give useful information on how to design biomaterials of polysaccharides such as HA, when the GJIC is an important function for evaluating biocompatibility of biomaterials.

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SAFETY EVALUATION OF TISSUE ENGINEERED MEDICAL DEVICES USING NORMAL HUMAN MESENCHYMAL STEM CELLS

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Abstract: For safety evaluation of tissue engineered medical devices using normal human mesenchymal stem cells (hMSC), in this study, some genes expressions in hMSC were compared with those in two kinds of the tumor cells (HeLa and HepG2). Effects of the passage number of hMSC on the gene expressions were also investigated using quantitative real-time RT-PCR. The proliferation speed of hMSC was lowered with the cell passage number. The mRNA expressions of c-myc oncogene and nucleostemin in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC). And the mRNA expressions of them in hMSC decreased with the passage number. Wnt-8B mRNA was expressed in the tumor cells (HeLa and HepG2), but not in the stem cells (hMSC) in any passage number. Although these results suggest change in these expression levels are not directly related to the tumorigenesis of hMSC, it is discussed that mRNA expression levels of c-myc oncogene, nucleostemin, and Wnt-8B can be used as an index of hMSC tumorigenesis.

Key words: hMSC, tumorigenesis, c-myc, nucleostemin, Wnt-8

1. INTRODUCTION

Several recent studies demonstrate the potential of tissue engineering for regenerative therapy using somatic stem cells. Human mesenchymal stem cells (hMSC) derived from bone marrow aspirates have the potentiality to differentiate into osteocytes, chondrocytes, myocytes, stromal cells, tenocytes, adipocytes, and so on. Therefore, the autologous cell or tissue transplantation using hMSC was noticed as the medical treatment under the various kinds of clinical conditions. On the

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other hand, owing to the similarity to the tumor cells, the stem cells also possess the ability of cell proliferation. Consequently, it is required to evaluate the safety of hMSC when that is used for tissue engineered medical devices. In this study, hMSC was compared with two kinds of the the tumor cells, HeLa (human cervix cancer) and HepG2 (human hepatoma), by investigating the differences in some genes expressions of each cells. Effects of the passage number of hMSC on the gene expressions were also investigated using quantitative real-time RT-PCR.

2. MATERIALS AND METHODS

Cell culture. Human mesenchymal stem cells (hMSC) purchased from the Cambrex Bio Science Walkersville, Inc. (MD, USA) was cultured in Mesenchymal Stem Cell Basal Medium (MSCBM; Cambrex Bio Science Walkersville, Inc.) supplemented with Mesenchymal Cell Growth Supplement (MCGS; Cambrex Bio Science Walkersville, Inc.), L-Glutamine and Pen/Strep at 37°C under a 5% CO_2 atmosphere. The cells was seeded at a density of 6,000 cells / cm^2 and subcultured when they are just sub-confluent (approximately 90% confluent) up to 10^{th} passage.

Quantitative RT-PCR. For quantitative RT-PCR, total RNA was extracted from hMSC of 1^{st}, 3^{rd}, 5^{th} and 10^{th} passage cultures with ISOGEN (NIPPON GENE CO., LTD.). RNA was then reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics; Tokyo, Japan). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, and Wnt-8B are summarized in Table 1. Amplifications of them were carried out for 10s at 95°C, for 15s at each annealing temperature, and for 12s at 72°C for 40 cycles. PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in Roche Light Cycler (software version 4.0).

Table 1. Primers and annealing temperatures used for Real time RT-PCR

Gene name	GenBank™ accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing Temp (°c)
c-myc	V00568	Forward	5'- GCG AAC ACA CAA CGT C -3'	1626	315	50
		Reverse	5'- CAA GTT CAT AGG TGA TTG CT -3'	1940		
nucleostemin	X91940	Forward	5'- CCA TTC GGG TTG GAG TAA -3'	782	284	50
		Reverse	5'- CTG TCG AGC ATC AGC C -3'	1065		
Wnt-8B	NM_014366	Forward	5'- AGT GAC AAT GTG GGC T -3'	331	244	60
		Reverse	5'- CGT GGT ACT TCT CCT TCA G -3'	574		

3. RESULTS

In this study, for safety evaluation of tissue engineered medical devices using normal human mesenchymal stem cells (hMSC), some genes expressions in hMSC were compared with those in two kinds of the tumor cells (HeLa and HepG2). At first, effect of the passage number on hMSC proliferlation was investigated. The proliferation speed of hMSC was lowered with the cell passage number (Fig. 1).

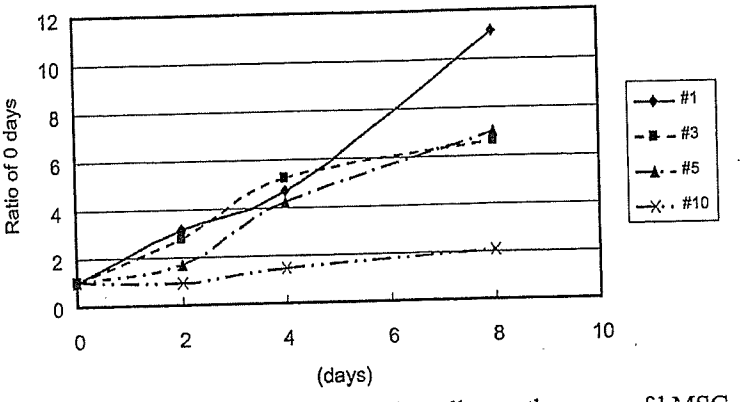

Fig. 1. Effect of the passage number on the cell growth curves of hMSC.

The mRNA expressions of c-myc oncogene in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC) (Fig. 2). The mRNA expressions of c-myc oncogene in hMSC in the 3rd and 5th passages were higher than in the 1st and 10th passages (Fig. 3). Similarly to c-myc, the mRNA expressions of nucleostemin in the tumor cells (HeLa and HepG2) were significantly higher than in the stem cells (hMSC) (Fig. 4). The mRNA expressions of nucleostemin in hMSC decreased with the passage number (Fig. 5). Wnt-8B mRNA was expressed in the tumor cells (HeLa and HepG2), but not in the stem cells (hMSC) in any passage number (Fig. 6).

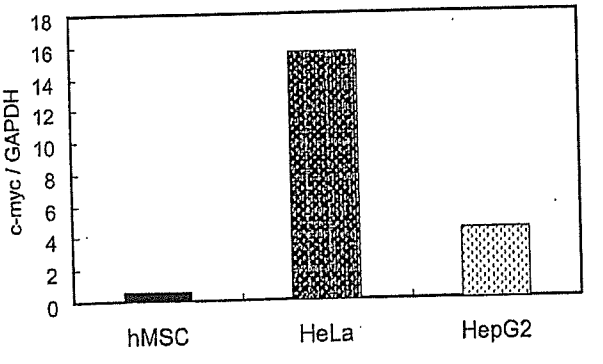

Fig. 2. The mRNA expression c-myc oncogene in hMSC, HeLa, and HepG2.