effects of PGA synthesized with and without an inorganic tin catalyst. In this study, the biocompatibility of PGA with and without a tin catalyst was investigated, using human articular chondrocytes (HAC) in a micromass culture system.

MATERIALS AND METHODS

Medium and polymers used for cell culture

Chondrocyte growth medium was obtained commercially from BioWhittaker (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (M_w = 1500) and without a catalyst (PGA) (M_w = 1100) were custom-made (TAKI chemicals, Kakogawa, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA).

Cells and culture methods

Human articular chondrocytes (HAC) of the knee joint was commercially obtained from BioWhittaker. High-density micromass cultures were started by spotting 4×10^5 cells in 20 μL of medium onto Costar 24-well tissue culture microplates (Costar type 3526, Corning). After a 2 h attachment period at 37°C in a CO_2 incubator, culture medium (1 mL/well) was added to each well. Media were supplemented with DMSO (0.8 μL/mL), PGA, and PGA(Sn) (50 μg/mL). HAC cultured with DMSO was used as the control. The cultures were continued for 4 weeks with a medium change twice a week. At least four cultures were performed for each sample.

Cell proliferation study

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[28] After the culture period, cells were fixed with 100% methanol at room temperature, followed by application of 0.1% crystal violet in methanol. After a proper wash, cells were again incubated in methanol; 100 μL from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm, using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

Cell differentiation assay was performed by alcian blue (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[29] Following crystal violet staining, the cells were washed with methanol and then 3% acetic acid. Cultures were then stained with 1% (v/v) alcian blue in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4*M* guanidine hydrochloride (GH), and the bound dye was measured at wavelength of 600 nm, using an ELISA reader (Bio-Tek Instruments). Fresh 4*M* GH served as the blank. Blank values were subtracted from experimental values to eliminate background readings.

Analytical assays

Commercially available assay kits (collagen and glycosaminoglycan [GAG] assay kits, Biocolor, Newtownabbey, Northern Ireland) were used for the measurement of collagen and sulfated GAGs within the cultured cells, as previously described.[30]

Briefly, for the GAG assay, GAG was extracted from the cultured cells using a solvent system of 4*M* guanidine-HCl, 0.5*M* sodium acetate, pH 6, with 1 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* *N*-ethylmaleimide (NEM). Incubation was carried out at 4°C on an orbital shaker for a 12- to 20-h period. After the extraction, the samples were centrifuged, and blyscan dye reagent (composed of 1,9-dimethyl methylene blue in an organic buffer) was mixed with the supernatant. The GAG–dye complex was collected by centrifugation. The dye bound to the pellet was subsequently solubilized by mixing it with a dissociation reagent. The absorbance of the samples was measured at a wavelength of 656 nm, using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen concentration (acid- and pepsin-soluble fractions) of the cultured chondrocytes was also measured. The acid-soluble collagen was removed by adding 0.5*M* acetic acid to the cultured cells, followed by centrifugation. The remaining pepsin-soluble collagen was subsequently extracted from the cultured cells. A pepsin solution (1 mg/10 mg tissue sample; Sigma) was added to the cells, and they were incubated overnight at 37°C. Both the acid- and pepsin-soluble collagen samples were further separated for assay by mixing with Sircol dye reagent for 30 min in a mechanical shaker, and the collagen–dye complex was collected by centrifugation. The dye bound to the collagen pellet was solubilized with an alkaline reagent, and the absorbance of the samples was measured at a wavelength of 540 nm, using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment.

Real-time polymerase chain reaction

To detect the presence of collagen type II and aggrecan, single-stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT), using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently, real-time polymerase chain reaction (PCR) was done using a LightCycler system with LightCycler FastStart DNA Master SYBR Green I

(Roche Diagnostics, Penzberg, Germany). The LightCycler™- Primer set (Roche Diagnostics) was used for quantitative detection of the collagen type II and aggrecan genes, and also for quantitation of a housekeeping gene, Glyceraldehide-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's instructions. An initial denaturation step at 95°C for 10 min was followed by amplification and extension steps for 35 cycles (95°C for 10 s, 68°C for 10 s, 72°C for 16 s) with final extension step at 58°C for 10 s. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Statistical study

Student's t tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation) except in figure 3. Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell proliferation

Chondrocyte proliferation was quantified by crystal violet staining and expressed as a percentage of the average control value (Fig. 1). Cell proliferation was increased 1.8-fold ($p < 0.05$) in PGA(Sn)-treated cultures compared with that of the control culture, whereas cell proliferation in PGA-treated cultures was almost identical to the DMSO-treated control culture.

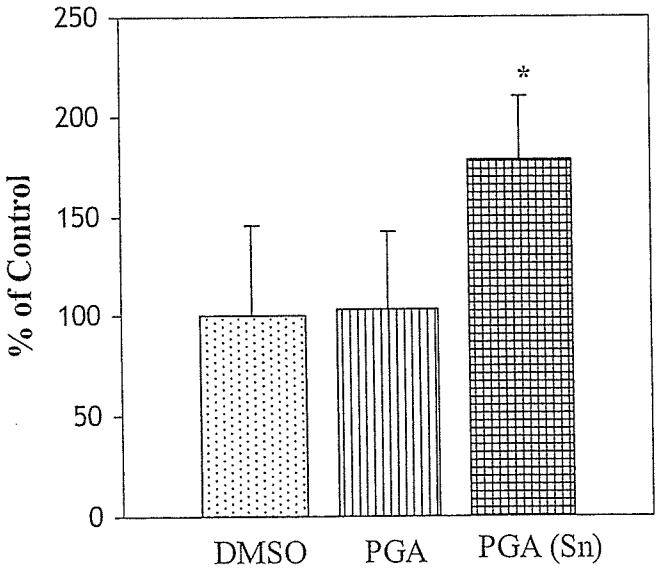

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

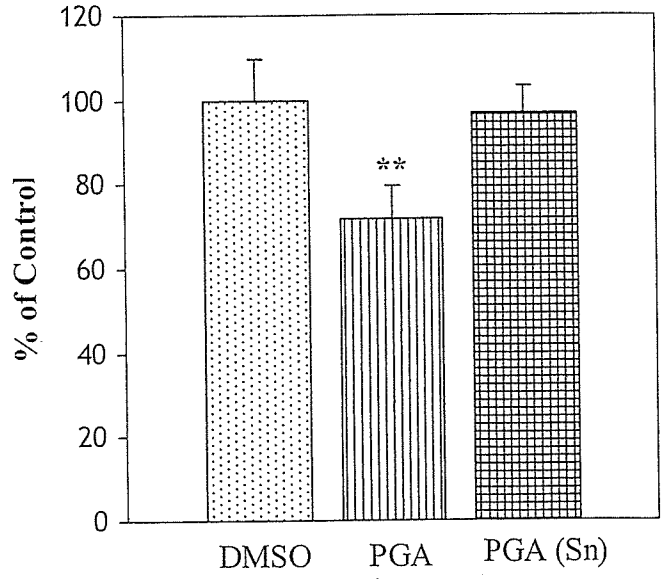

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

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

Extracellular matrix gene expression

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

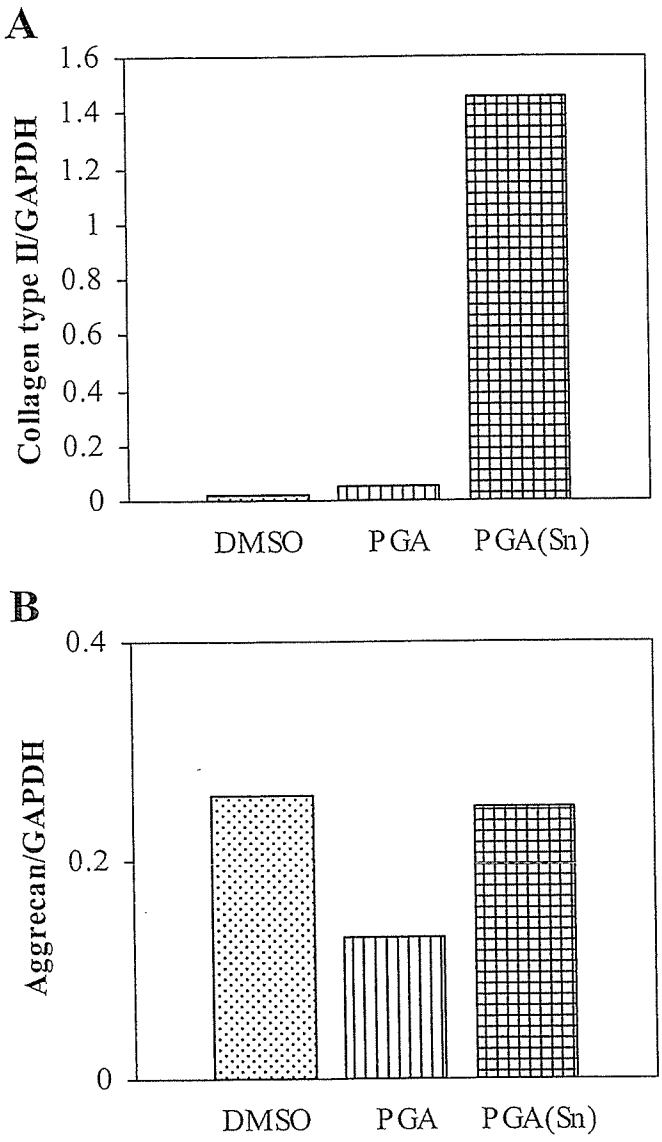

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between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

Measurement of collagen type II protein

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

Measurement of total collagen

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

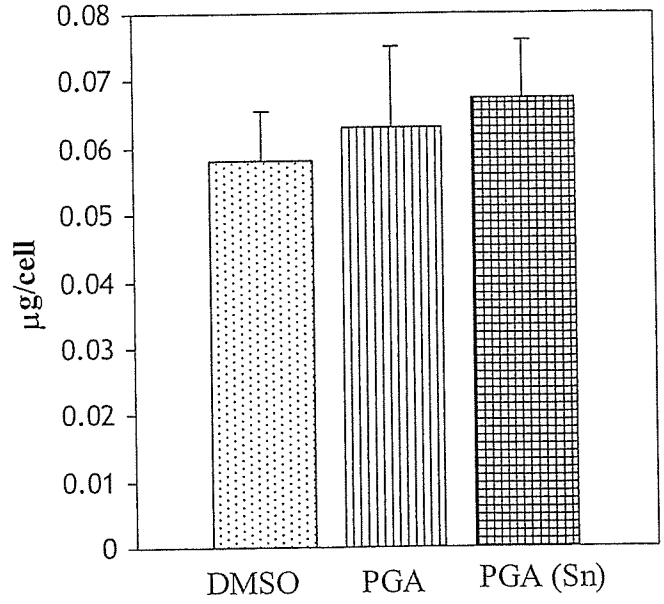

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(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-

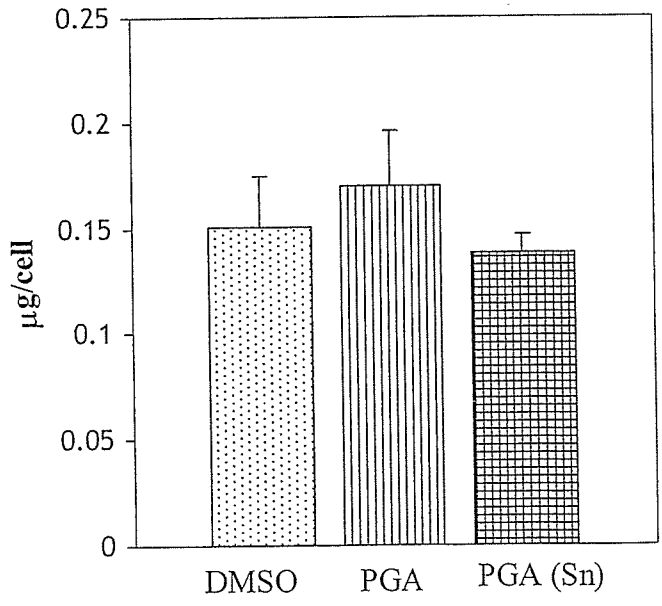

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.

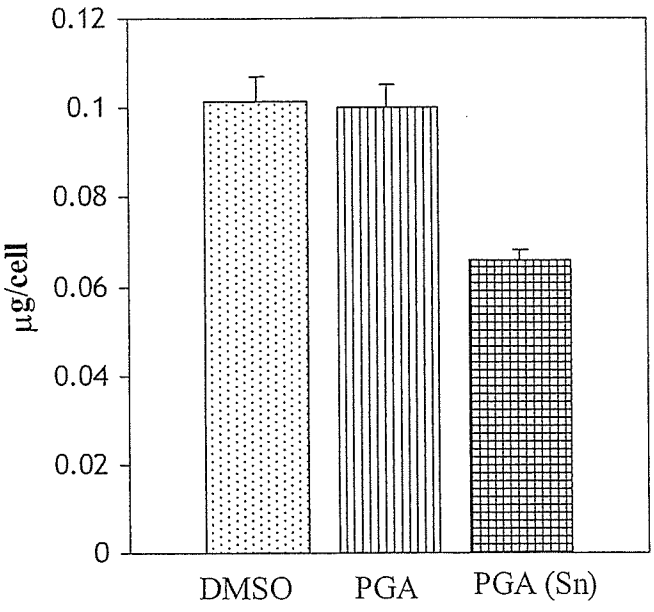

Figure 6. Evaluation of sulfated GAG. There was decrease in the amount of sulfated GAG in PGA(Sn)-treated cultured cells compared with that of the control. All experiments were run in quadruplicate for two separate times.

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

of PGA showed enhancing effects of tin compounds on chondrocytes in comparison to without tin polymer because of increase in the permeability of inorganic tin under the presence of PGA. However, further study is required for the application of this PGA(Sn) in clinical practice.

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The effect of hyaluronic acid on insulin secretion in HIT-T15 cells through the enhancement of gap-junctional intercellular communications

Yuping Li[a,1], Tsutomu Nagira[a,b], Toshie Tsuchiya[a,*]

[a]*Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b]*Japan Association for the Advancement of Medical Equipment, 3-42-6 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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Abstract

The transplantation of bioartificial pancreas has the potential to restore endogenous insulin secretion in type I diabetes. The bioartificial pancreas is constructed in vitro from cells and a support matrix. Hyaluronic acid (HA) is an extremely ubiquitous polysaccharide of extracellular matrix in the body and plays various biological roles. It has been suggested that high molecular weight (HMW) HA increases in the function of gap-junctional intercellular communications (GJIC) and the expression of connexin-43 (Cx43). To determine whether the function of pancreatic β-cells is affected by gap junctions after HMW HA-treatment, we exposed HIT-T15, a clonal pancreatic β-cell line, in various concentrations of HA for 24 h, and then detected the insulin secretion and content, 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 function, the increase of insulin release and insulin content. The results obtained in this study suggest that HA-coating increases the insulin secretion of HIT-T15 cells by the enhancement of Cx43-mediated GJIC. The results give useful information on design biocompatibility of HA when is used as a biomaterial for bioartificial pancreas.

Keywords: Hyaluronic acid; Gap-junctional intercellular communications; HIT-T15 cells; Insulin; Bioartificial pancreas

1. Introduction

Type I diabetes is caused by the autoimmune destruction of the β-cells. All patients with type I diabetes require daily insulin shots for the control of glucose levels. However, the insulin therapy cannot inhibit the development of serious chronic complications. The pancreas transplantation has been expected to be the most promising approach toward treating diabetes. The bioartificial pancreas is constructed in vitro from insulin-secreting cells or islets and a support matrix by a tissue engineering method. The frequently used matrix materials are alginate and agar [1,2]. Although bioartificial pancreatic constructs contain insulin-secreting cells entrapped in agar or alginate matrix implanted into the peritoneal cavity of the diabetic patient, mice, and dog, can restore normoglycemia and markedly abate diabetic symptoms, there are important questions in the structural integrity of support matrix, metabolic activity and viability of cells or islets, and late vascular thrombosis [1,2]. Therefore, the new matrix biomaterials, which mimic the functions of extracellular matrix (ECM), need to be researched.

Hyaluronic acid (HA) is an extremely ubiquitous member of the nonsulfated glycosaminoglycan ECM molecule family and is thought to play various biological roles particularly in growth, adhesion, proliferation, differentiation, and cell migration [3,4]. More importantly, the receptor for HA-mediated motility regulates gap-junction

*Corresponding author. Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 3 3700 9196; fax: +81 3 3700 9196.
E-mail address: tsuchiya@nihs.go.jp (T. Tsuchiya).
[1]Present address: School of Life Sciences, Nanchang University of Sciences and Technology, Nanchang, China.

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channel and connexin-43 (Cx43) expression by its actions on focal adhesions and the associated cytoskeleton [5]. In addition, Park and Tsuchiya [6] have reported that high molecular weight (HMW) HA-coating can enhance the function of gap-junctional intercellular communications (GJIC). The insulin secretion from pancreatic β-cells is a multicellular event depending on their interaction with neurotransmitters and numerous signal molecules carried by blood and also direct interactions between cell–cell and cell–matrix contacts by gap-junctional channels, which mediate exchanges of molecules smaller than 1000 Da, such as ions, small metabolites, and second messengers between adjacent cells. The latter interactions are thought to be crucial regulatory mechanisms of insulin secretion [7–9], and the pharmacological blockade of GJIC markedly decreases insulin release [8]. However, the effects of HMW HA as biomaterials of support matrix on functions of pancreatic β-cells and gap-junctional channel remain unclear.

In the present study, we investigated the effects of HMW HA on the function of GJIC, the expression of Cx43, insulin content, and insulin secretion using HIT-T15 cells in vitro. These results suggest that HMW HA can be used as the biomaterial for the development of a bioartificial pancreas: design biocompatibility of HA depends on the molecular-weight size of HA, and its application method and concentration.

2. Materials and methods

2.1. Materials

Lucifer yellow was purchased from Molecular Probes (Eugene, OR). HA (1680 kDa) and TetraColor ONE (WST-8) were supplied by Seikagaku Industries, Ltd. (Tokyo, Japan). ELISA insulin assay kit was obtained from Morinaga Seikagaku Co. (Yokohama, Japan). Bovine serum albumin (BSA) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4), fetal bovine serum (FBS), and anti-Cx43 were purchased from Sigma Chemical Co. (St. Louis, MO). β-actin antibody was obtained from Cell Signaling Technology Inc. (Tokyo, Japan). Roswell Park Memorial Institute (RPMI) 1640 medium was from Nissui pharmaceutical Co. (Tokyo, Japan). All other chemicals used were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of media and culture dishes

The HA polysaccharide was dissolved in distilled water at a concentration of 4 mg/ml. Each of the 35-mm culture dish (Falcon 1008, Becton Dickinson) was coated at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 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, different media were prepared at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. HA-treatment is performed to cells for 24 h.

2.3. Cells and cell culture

A hamster pancreatic β-cell line, HIT-T15 (HIT-T15 cells, Dainippon Pharmaceutical Co., Japan), was cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 IU penicillin-G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The subculture 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 replaced every 2 days.

2.4. Measurement of cell viability

To evaluate the affect of HMW HA on cell viability of HIT-T15 cells, HIT-T15 cells (1×10^5) were incubated into the various concentrations of HA-coated 24-well plates, 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 24 h of HA-treatment, the cell viability was determined by the WST-8 reduction assay, according to the manufacturer's instructions. Control cells received fresh medium without HA.

2.5. Measurement of insulin release and insulin content

HIT-T15 cells were treated as described above. After pre-incubating for 30 min at 37 °C in KRB buffer, no glucose cells were stimulated for 60 min with 11.1 mM glucose in KRB buffer. The medium was collected, centrifuged for 5 min at 3000*g*, and the supernatant was frozen at −80 °C for insulin release assay. Cultures were then extracted for 24 h at 4 °C in acid-ethanol and the extracts also frozen for determination of insulin and protein content. Insulin was determined by ELISA insulin kit with rat insulin as standard, according to the manufacturer's instructions. Protein content was measured by the BCA protein assay reagent kit with albumin as standard (PIERCE). Values of secreted insulin were normalized to protein content.

2.6. Measurement of dye transfer

Gap junction-mediated communication between β-cells regulates the insulin secretion and insulin biosynthesis. Because HMW HA-coating increased the insulin release and insulin content but not HA-added, we tested whether the HA-coating increases the insulin secretion and insulin content have a relationship with gap junctions between HIT-T15 cells. HIT-T15 (5×10^5) cells were exposed to the HA-coated (0.1, 0.25, and 0.5 mg/dish) 35-mm glass coverslip (Ashland, MA) and incubated for 24 h to evaluate dye coupling using Lucifer yellow. The cells were rinsed with phosphate-buffered saline [PBS(+)] containing Ca^{2+}/Mg^{2+}, and 3 ml of PBS(+) containing 1% BSA and 10 mM HEPES (pH 7.4) were added to keep a sufficient pH stability under the microscope. The junctional coupling of HIT-T15 cells was determined by injecting Lucifer yellow into individual cells within monolayer clusters. Injections were performed on a phase-contrast microscope with InjectMan NI2 and microinjector FemtoJet (Eppendorf AG, Germany) using glass micropipette that were filled with a 4% solution of Lucifer yellow CH (MW 457.2) dissolved in 0.33 M lithium chloride, as previously described [11]. An injection pressure of 6.5 psi for 200 ms was used for each injection. The coupling extent was evaluated by counting dye-transferred cells at 2 min after microinjection. There was no leakage of injected dye into the medium.

2.7. Western blot analysis

HIT-T15 cells were grown into the various concentration of HA-coated 100-mm plastic dishes (0.1, 0.25, and 0.5 mg/dish) (FALCON 3003; Falcon) for 24 h, rinsed with Ca^{2+}/Mg^{2+}-free PBS(−) and then lysed in CelLytic™-M lysis/extraction reagent (Sigma). Protein content was measured by the BCA protein assay reagent kit (PIERCE). Samples of total extracts (20 μg protein/lane) were fractionated by eletrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The contents of the gels were transferred to PVDF membranes (Clear Blot Membrane-P). Membranes were saturated for 2 h at room temperature in Block Ace (Dainippon Pharmaceutical Co.,

Japan) and then were incubated with antibodies directed against Cx43 (1:1000) and β-actin (1:1000) as the primary antibody overnight at 4 °C. After repeated rinsing in PBS–Tween, the immunoblots were incubated with a peroxidase-conjugated antibody against rabbit (1:5000) at room temperature for 1 h. Membranes were developed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

3. Results

3.1. Cell viability

In order to evaluate the affect of HMW HA on cell viability, HIT-T15 cells were incubated with HA-coated (0.01, 0.05, 0.1, 0.5, and 1.0 mg/dish) or -added (0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml) for 24 h. After 24 h exposure to HA-added, there was no significant change in the viable HIT-T15 cell number at the low concentration of HA-added ($\leqslant$1.0 mg/dish) compared to control. In contrast, after 24 h of incubation, the cell viability of HIT-T15 cells grown on high concentration HA-coated dishes ($\geqslant$1.0 mg/dish) was significantly less than on low concentration HA-coated and control (Fig. 1). Therefore, all further studies were conducted using low concentration of HA ($\leqslant$0.5 mg/dish).

3.2. Insulin secretion and insulin content

HIT-T15 cells, retain glucose-stimulated insulin secretion, showed an increase in insulin secretion as a function of stimulation. Thus, their insulin output was 2.73±0.36 and 3.90±0.41 pg/μg protein in the base and glucose-stimulation (11.1 mM), respectively ($n = 9$ dishes from three independent experiments). When these cells were exposed to a low concentration of HA-coating (0.1, 0.25, and 0.5 mg/dish) for 24 h, their insulin secretion was significantly increased in the presence of glucose-stimulation (Fig. 2). However, in contrast, when HIT-T15 cells were incubated with HA-addition for 24 h, the increasing effect was not exhibited. The insulin secretion was without a difference between control and HA-addition (Fig. 2). On the other hand, after acid-ethanol extraction, we found that the insulin content of the HIT-T15 cells grown onto the HA-coated dishes was significantly increased but not HA-added (Fig. 3).

GJIC and Cx43 are thought to be crucial regulatory mechanisms of insulin secretion and insulin content. As described above, HA-coating increased insulin secretion and insulin content of the HIT-T15 cells. In addition, Park and Tsuchiya [6] reported that HMW HA-coating can enhance the function of GJIC in normal human dermal fibroblasts but not HA-addition. Hence, all further studies on the mechanism of insulin secretion and insulin content were conducted using HA-coating.

3.3. Dye transfer

We assessed the function of GJIC using Lucifer yellow by counting the number of dye-transferred cells at 2 min

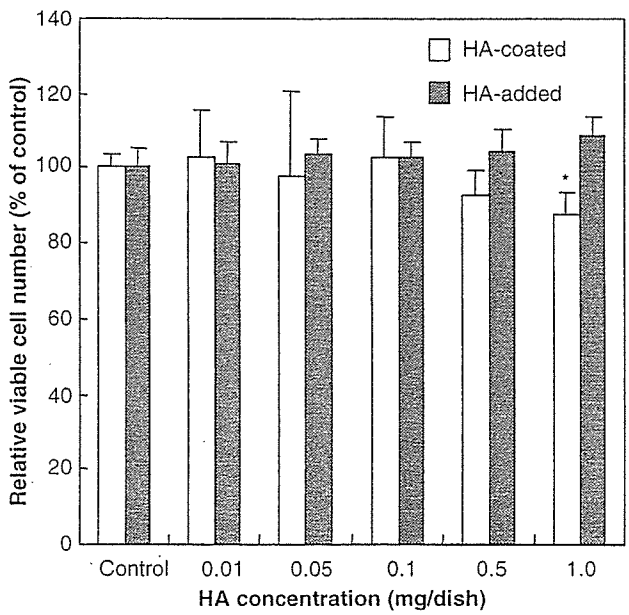

Fig. 1. Concentration-dependent effects of HA-treatment on viability of HIT-T15 cell. After HIT-T15 cells were incubated with HA-coated or HA-added for 24 h, the viable cell numbers of HIT-T15 cell were determined by WST-8 assay as described in methods. Each value denotes the mean±S.D. of three separate experiments. *$P \leqslant 0.05$ compared to control under the HA-coated condition.

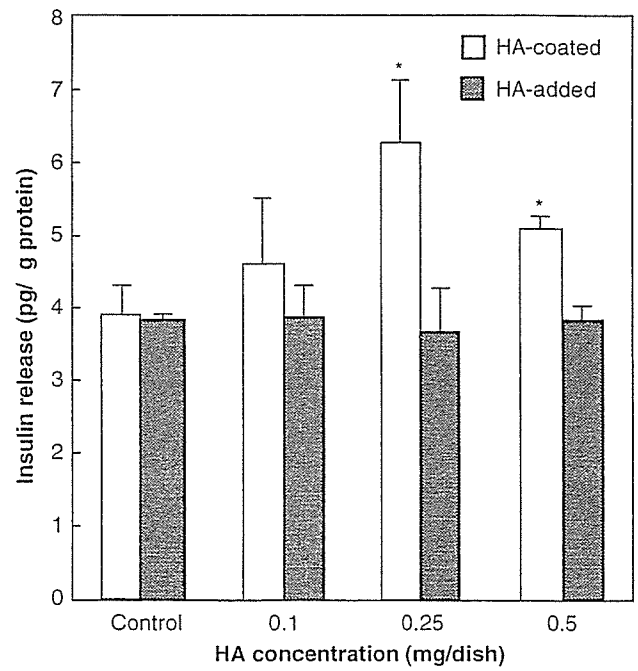

Fig. 2. Insulin secretion from HIT-T15 cells by HA-treatment. HIT-T15 cells were incubated with HA-coating (□) or HA-added (■) for 24 h and then stimulated for 60 min with 11.1 mM glucose in KRB buffer. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean±S.D. of three separate experiments. *$P \leqslant 0.05$, compared to control in the presence of glucose.

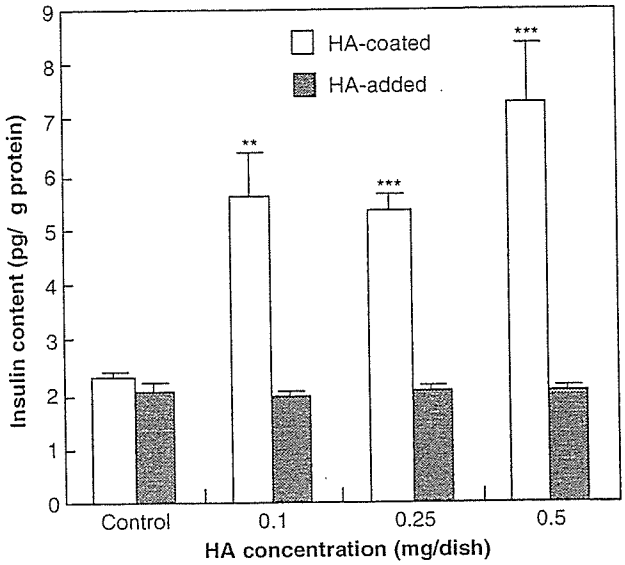

Fig. 3. Insulin content of HIT-T15 cells incubated with HA-coated (□) and HA-added (■). Cells were incubated in the presence of different HA concentrations (0.1–0.5 mg/dish) for 24 h and then stimulated for 60 min with 11.1 mM glucose. The insulin content in the extracts was determined by ELISA insulin kit. Each value denotes the mean±S.D. of three separate experiments. $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$ compared to control.

after microinjection. Fig. 4A shows the patterns of dye transfer in HIT-T15 cells treated with HA-coating (0.1, 0.25, and 0.5 mg/dish) for 24 h. Most microinjections led to the intercellular transfer of Lucifer yellow, indicating the frequent coupling of HIT-T15 cells. Under control conditions, microinjection experiments revealed that 47.1% of HIT-T15 cells transferred Lucifer yellow with a limited number (1.5±0.6) of microinjection cells. In HA-coated conditions, almost injected cells (95%) showed Lucifer yellow dye transfer, the number of Lucifer yellow-transferred cells (3.2±1.3, 4.4±1.9, and 4.1±1.9, respectively) was more than that of the control condition ($P<0.001$) (Fig. 4B), which indicated that GJIC function was activated by the HA-coating.

3.4. Cx43 expression

Cx43 is the 43-kDa member of a conserved family of membrane spanning gap-junction proteins. To provide further evidence that the HA-coating increased the function of GJIC, relative to the levels of actin, comparable levels of immunolabeled Cx43 was detected in 0.1, 0.25, and 0.5 mg/dish of HA-coating cells. Whole cell lysates from HA-coated dish were subjected to SDS–PAGE. Immunoblot analysis was performed with an antibody that specifically recognized Cx43 or β-actin. A Western blot analysis revealed that Cx43 proteins are present in cultured HIT-T15 cells in three forms at 43 kDa region, consisting of a nonphosphorylated form and phosphorylated forms (P1 and P2). HA-coating appeared to induce a greater concentration-dependent increase in all three Cx43 protein levels than control. However, the protein level of β-actin was no different from them (Fig. 5), indicating HA-coating increases the function of GJIC via the expression of Cx43. To account for differences in loading, proteins were both stained with Coomassie blue and immunolabeled for β-actin. The latter staining, which did not change in our experiments relative to that of Coomassie blue (data not shown), was used as an internal standard. These results suggested that HA-coating specifically increased the Cx43 protein but not all cell proteins of HIT-T15 cells.

4. Discussion

The transplantation strategy of bioartificial pancreas is to construct bioartificial tissues in vitro from cells or islets and a support matrix and implant the construct into the body in place of the original. The support matrix must be able to maintain the functions of differentiated cells or contain and/or be able to release appropriate biological signaling information to promote and maintain cell adhesion and differentiation. HA is a high-molecular-mass polysaccharide of support matrix in the body, which is believed to play roles in maintaining various physiological functions including water and plasma protein homeostasis, cell proliferation, cell locomotion, and migration [3]. HA is plentiful, easy to extract and mold into a variety of shape, and biodegradable. It is thus widely used matrix biomaterial for bioartificial tissues [10]. In this study, we investigated whether administration of various concentration of HMW HA influences the viability, GJIC, and insulin secretion of pancreatic β-cells as a matrix biomaterial of bioartificial pancreatic constructs.

Previous study has shown that HMW (310 and 800 kDa) HA-coating (2.0 mg/dish) resulted in low adhesiveness to the cells and the decrease of viability in normal human dermal fibroblasts, because of the change in GJIC functions and induction of various genes including cytokines, adhesion molecules, and growth factors [6,11,12]. In the present study, similar results were obtained. After 12 h, the HIT-T15 cells grown into low concentration HA-coated dishes (0.1, 0.25, and 0.5 mg/dish) and control cells already had attached and confluent but not high concentration HA-coated dishes (≥1.0 mg/dish). We showed that treatment with high concentration of HMW (1680 kDa) HA-coated dose dependently inhibited the viability of HIT-T15 cells. In contrast, there was no difference in viability of HIT-T15 cells between the control and HA-added dishes. These results indicated that among the individual qualities of ECM, the viscosity plays a decisive role. The changes of cell viability by HA-treatment may depend on the cell attachment activity. The difference in cell attachment activity may depend on the surface structure of the coated HA, because the HMW HA-coated surface provides a stable anionic surface that prevents cells attachment at the early time [13]. This result suggests that the molecular-weight size of HA and its

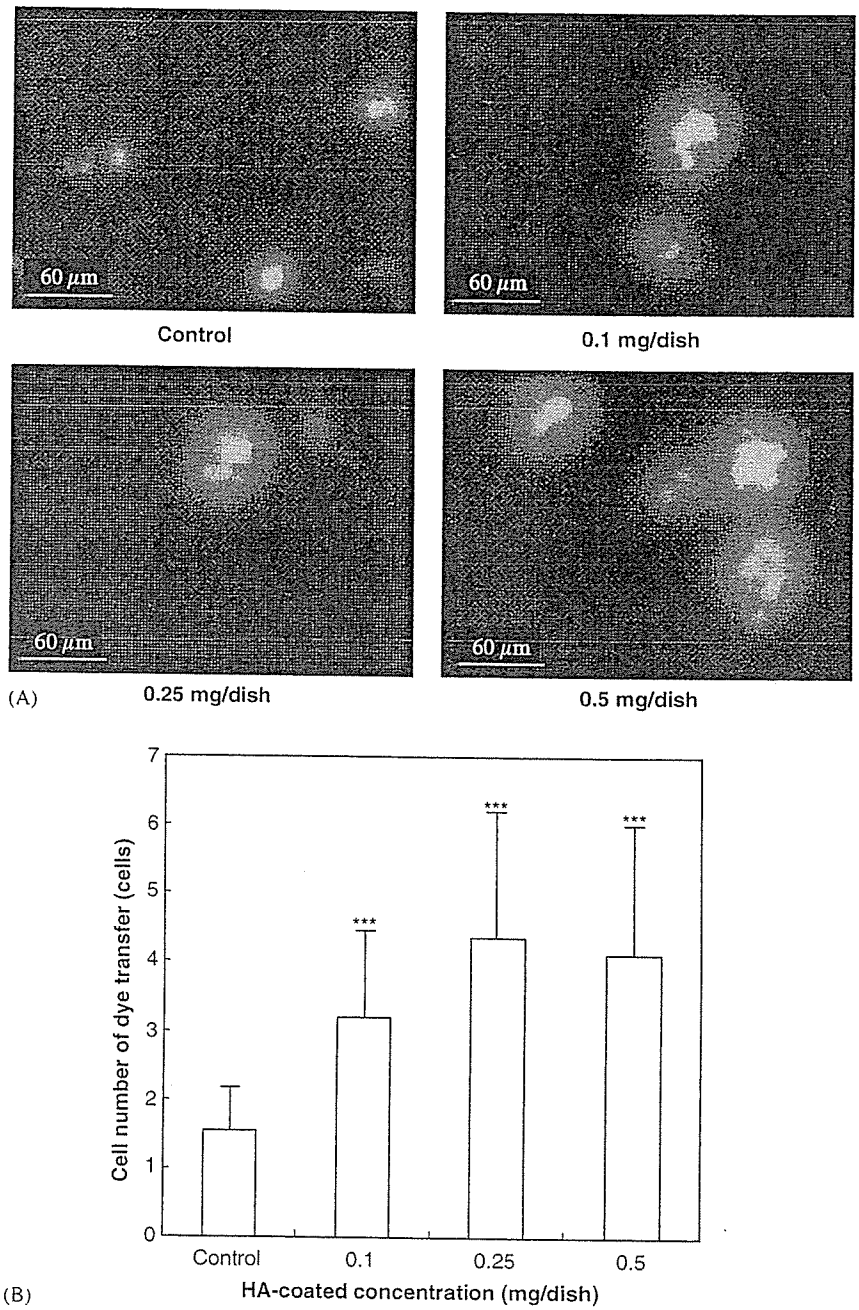

Fig. 4. Concentration-dependent effects of HA-coating on dye transfer in HIT-T15 cells. Cell adherent to glass coverslips were microinjected with 4% Lucifer yellow. Transfer of dye to neighboring cells was assessed by epifluorescence microscopy 2 min later. This is a representative expression of 18 injections per group (A). The number of neighboring cells that received dye was quantified (B). Each value expressed as the mean ± S.D. ($n = 18$). $^{***}P \leqslant 0.001$ compared to control.

application method and concentration are important factors for generating biocompatible tissue-engineered products.

It has been reported that single β-cells (which cannot form gap junctions) show alterations in both basal and stimulated release of insulin, in protein biosynthesis, and in the expression of the insulin gene. The sustained stimulation of insulin release is associated with an increase in β-cells coupling, in the expression of gap junctions by a unique mechanism for direct equilibration of ionic and molecular gradients between nearby cells [14–16]. In this study, we found that the insulin release and insulin content are increased and GJIC activity was enhanced in cultured HIT-T15 cells by low concentration HMW HA-coating in spite of the inhibitory effects on the cell viability in high concentration HA-coating dishes. This finding was consistent with previous reports. The effect of HA may be influenced by the viscosity of HA, the concentration of

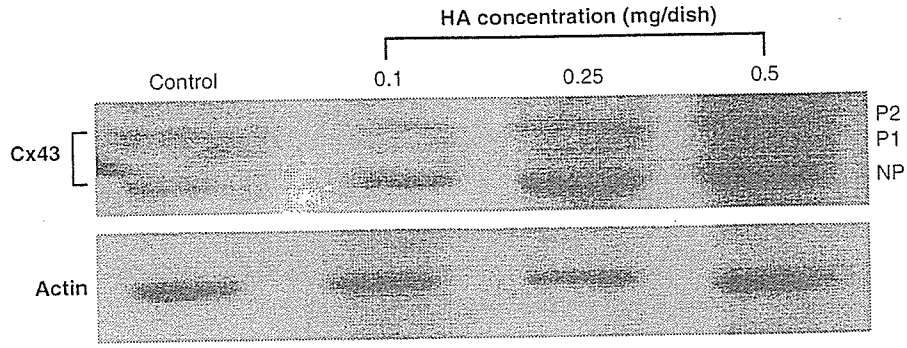

Fig. 5. Identification of Cx43 in HIT-T15 cells grown on the HA-coating dish by Western blot analysis. After HIT-T15 cells were incubated into HA-coated dish for 24 h, cells were lysed and proteins (20 μg) were separated by SDS–PAGE followed by Western blotting using rabbit anti-Cx43 antibody. Actin immunostaining was used to assess equivalent protein loading. This is a representative autoradiogram of three experiments.

FBS and the nutrients in media such as hormone, growth factor (FGF, etc.), cell adhesion molecule (N-CAM and cadherins), and transportation protein [6,17]. As a result, the HIT-T15 cells can use these nutrients and the nutrient-enriched substrata (e.g. natural ECMs) by ionic interaction and the binding of HMW HA to various kinds of cytokines, to change the cell aggregations, resulting in the increase of GJIC. With the evidence above, the enhancement of GJIC activity induced by HA-coating participated in the regulation of insulin release and insulin biosynthesis. On the other hand, the glucose stimulus-secretion coupling in β-cells generated several signals, including a signal to secrete preformed insulin stored in secretory vesicles, a signal, which may be the same or different, to secrete newly made insulin, and a signal to synthesize more insulin. The mechanism of glucose-induced insulin secretion is distinct from that of glucose-induced proinsulin biosynthesis and insulin gene transcription [18]. Moreover, the qualities of ECM affect the insulin release [19]. Therefore, it is possible that HA-coated dishes promoted a large increase in insulin synthesis but only a modest increase in insulin release. The detailed action mechanism should be investigated in the next study.

In native and tumoral insulin-producing pancreatic β-cells, gap-junction protein Cx43 has been identified. Furthermore, the stable transfection of the gene coding for Cx43 induces the expression of functional gap-junction channels and improves both the biosynthetic and secretory defects of the cells. Cx43-transfection and incidence of junctional coupling also secrete more insulin than wild-type and noncommunicating cells, the absence of Cx43 implicated in the loss of β-cell-specific functions in vitro and in vivo [9,14]. In this study, HA-coating expressing high levels of the Cx43, gap junctions, and coupling, showed the striking enhancement of the amounts of stored hormone in HIT-T15 cells and promoted the glucose-induced insulin release, indicating that adequate levels of Cx43 and coupling are required for proper insulin production. These results provide further evidence that HA-coating increases the pancreatic β-cells function by enhancing the function of Cx43-mediated GJIC.

5. Conclusion

In conclusion, the function of GJIC is considered to be a useful marker for evaluating tissue-engineered products. The data obtained in this study show 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. The growth regulation with a bioartificial pancreatic construct using HA is achievable. These results give useful information on design biocompatibility of HA when the HA is used as a biomaterial for bioartificial pancreas. HA-coating may be a new technique for constructing three-dimensional bioartificial pancreas in tissue engineering.

Acknowledgements

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INCREASE IN THE INSULIN SECRETION OF HIT-T15 CELLS:

Gap Junctional Intercellular Communications Enhanced by Hyaluronic Acid

Yuping Li, Tsutomu Nagira and Toshie Tsuchiya
Division of Medical Devices, National Institute of HealthScience, Kamiyaga 1-18-1, Setagaya-ku, Tokyo, Japan

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 ($\geqq$ 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.

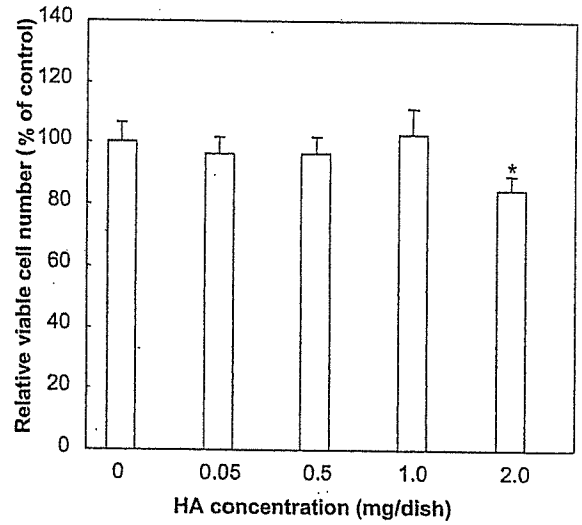

Figure 1. Viability of HIT-T15 cell after 72 h of HA-treatment. The viable cell numbers of HIT-T15 cell were determined by alamarBlue™ assay as described in Section 2. Each value denotes the mean ± S.D. $^*P \leq 0.05$ compared to untreated control.

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.

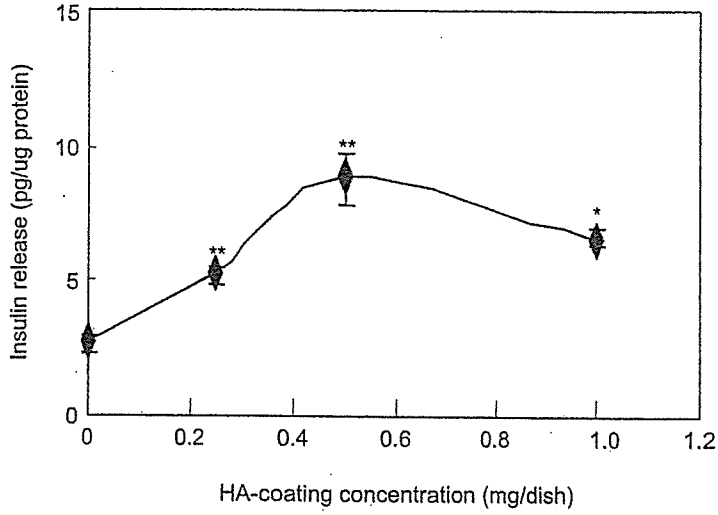

Figure 2. Concentration-dependent effects of HA-coating on insulin secretion from HIT-T15 cells. Treated with HA for 72 h, HIT-T15 cells were incubated with KRB buffer for 60 min. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean ± S.D. of three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

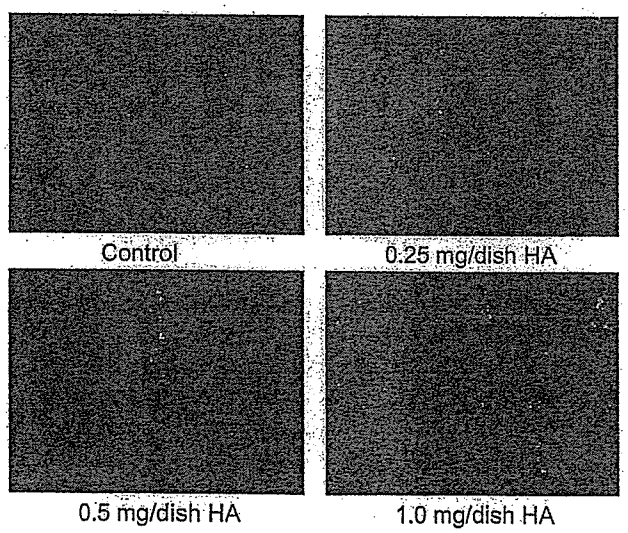

Figure 3. Time-course effects of various concentrations of HA-coating on the dye transfer ratio of HIT-T15 cells.

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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EFFECT OF BIODEGRADABLE POLYMER POLY (L-LACTIC ACID) ON THE CELLULAR FUNCTION OF HUMAN ASTROCYTES

Naohito Nakamura and Toshie Tsuchiya
Departmetn of Medicdal Devices, National Institute of Health Science; Kamiyoga 1-18-1 Setagaya-ku, Tokyo, Japan

Abstract: The objective of this study is to assay the efficiency and safety of poly (L-lactic acid) (PLLA) on human neural tissues. We used normal human astrocytes (NHA) to clarify effects of PLLA on their proliferation and differentiation. We cultured NHA with PLLA for one week, and determined NHA cell number and neural cell specific marker genes to assay their proliferation and development, respectively.

Cell proliferation was determined by tetrazolium salt (MTT) assay. The cell number of astrocytes cultured with 50 μg/ml PLLA was 70% of control. It has been suggested that a part of astrocytes had neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte. We compared gene expression of neural cell specific markers. Expression of Nestin, a specific gene for neural precursor cell was decreased in a dose-dependent manner, while expression of specific genes for neuron markers and astrocyte markers were not different from that of control.

PLLA suppressed astocyte proliferation in dose dependent manner. A neural precursor cell marker decreased when astrocytes were cultured with PLLA. These findings suggest that PLLA reduces proliferation and developmental potential of astrocytes.

Key words: Astrocyte, PLLA, proliferation, development

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