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.

References

1. Yasui N, Osawa S, Ochi T, Nakashima H, Ono K. Primary culture of chondrocytes embedded in collagen gels. Exp Cell Biol 1982;50:92–100.
2. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;186:231–239.
3. Grandolfo M, D'Andrea P, Paoletti S, Martina M, Silvestrini G, Bonucci E, Vittur F. Culture and differentiation of chondrocytes entrapped in alginate gels. Calcif Tissue Int 1993;52:42–48.
4. Loty S, Sautier JM, Loty C, Boulekbache H, Kokubo T, Forest N. Cartilage formation by fetal rat chondrocytes cultured in alginate beads: a proposed model for investigating tissue-biomaterial interactions. J Biomed Mater Res 1998;42:213–222.
5. Perka C, Spitzer RS, Lindenhayn K, Sittinger M, Schultz O. Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. J Biomed Mater Res 2000;49:305–311.
6. Meinhart J, Fussenegger M, Hobling W. Stabilization of fibrin-chondrocyte constructs for cartilage reconstruction. Ann Plast Surg 1999;42:673–678.
7. Perka C, Schultz O, Lindenhayn K, Spitzer RS, Muschik M, Sittinger M, Burmester GR. Joint cartilage repair with transplantation of embryonic chondrocytes embedded in collagen-fibrin matrices. Clin Exp Rheumatol 2000;18:13–18.
8. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000;21:1145–1153.
9. George-Weinstein M, Gerhart JV, Foti GJ, Lash JW. Maturation of myogenic and chondrogenic cells in the preosmitic mesoderm of the chick embryo. Exp Cell Res 1994;211:263–274.
10. Ashammakhi N, Rokkanen P. Absorbable polyglycolide devices in trauma and bone surgery. Biomaterials 1997;18:3–9.
11. Middleton JC, Tipton JA. Synthetic biodegradable polymers as orthopedic devices. Biomaterials 2000;21:2335–2346.
12. Kohn J, Langer R. Bioresorbable and bioerodible materials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemon JE, editors. An Introduction to Materials in Medicine. San Diego: Academic Press; 1997. p 65–73.
13. Wong WH, Mooney DJ. Synthesis and properties of biodegradable polymers used as synthetic matrices for tissue engineering. In: Atala A, Mooney D, editors. Synthetic Biodegradable Polymer Scaffolds. Boston: Burkhauser; 1997. p 51–84.
14. Yaszemsky MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: Molecular, cellular and tissue strategies to engineer human bone. Biomaterials 1996;17: 175–185.
15. Burg KJL, Porter S, Kellam JF. Biomaterials development for bone tissue engineering. Biomaterials 2000;21:2347–2359.
16. Tessa H, Sundback C, Hunter D, Cheney M, Vacanti JP. A polymer foam conduit seeded with Schwann cells promoted guided peripheral nerve regeneration. Tissue Eng 2000;6:119–127.
17. Bryan DJ, Holway AH, Wang KK, Silva AE, Trantolo DJ, Wise D, Summerhayes JC. Influence of glial growth factor and Schwann cells in a bioresorbable guidance channel on peripheral nerve regeneration. Tissue Eng 2000;6:129–138.
18. Evans GRD, Brandt K, Widmer MS, Lu L, Meszlenyi RK, Gupta PK, Mikos AG, Hodges J, Williams J, Gurlek A, Nabawi A, Lohman R, Patrick JCR. In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 1999;20:1109–1115.
19. Matsumoto K, Ohnishi K, Sekine T, Ueda H, Yamamoto Y, Kiyotani T, Nakamura T, Endo K, Shimizu Y. Use of a newly developed artificial nerve conduit to assist peripheral nerve regeneration across a long gap in dogs. ASAIO J 2000;46:415–420.
20. Salanki Y, D'eri Y, Platokhin A, Sh-Rozsa K. The neurotoxicity of environmental pollutants: the effect of tin (Sn2+) on acetylcholine-induced currents in growing pond snail neurons. Neurosci Behav Physiol 2000;30:63–73.
21. Gyori J, Platoshyn O, Carpenter DO, Salanki J. Effect of inorganic and organic tin compounds on Ach- and voltage-activated Na currents. Cell Mol Neurobiol 2000;20:591–604.
22. Chang LW. The neurotoxicology and pathology of organomercury, organolead, and organotin. J Toxicol Sci 1990;15:125–151.
23. de Mattos JC, Dantas FJS, Bezerra RJAC, Bernardo-Filho M, Cabral-Neto JB, Lage C, Leitao AC, Caldeira-de-Araujo A. Damage induced by stannous chloride in plasmid DNA. Toxicol Lett 2000;116:159–163.
24. Chao JS, Wei LY, Huang MC, Liang SC, Chen HH. Genotoxic effects of triphenyltin acetate and triphenyltin hydroxide on mammalian cells in vitro and in vivo. Mutat Res 1999;21:167–174.
25. De Santiago A, Aguilar-Santelises M. Ogranotin compounds decrease in vitro survival, proliferation and differentiation of normal human B lymphocytes. Hum Exp Toxicol 1999;18:619–624.
26. Yamaguchi M, Kitade M, Okada S. The oral administration of stannous chloride to rats. Toxicol Lett 1980;5:275–278.
27. Chang LW. Hippocampal lesions induced by trimethyltin in neonatal rat brain. Neurotoxicology 1984;5:205–215.
28. Tsuchiya T, Ikarashi Y, Arai T, Ohhashi J, Nakamura A. Improved sensitivity and decreased sample size in a cytotoxicity test for biomaterials: a modified colony microassay using a microplate and crystal violet staining. J Appl Biomater 1994;5: 361–367.
29. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable polymers. Tissue Eng 2001;7:781–790.
30. Brown AN, Kim BS, Alsberg E, Mooney DJ. Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs. Tissue Eng 2000;6:297–305.
31. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987;237:1588–1595.
32. William SDF. Some observations on the role of cellular enzymes in the *in vivo* degradation of polymers. In: Syrett BC, Acharya A, editors. Corrosion and degradation of implant materials 1979. p 61–75.
33. Webber RJ, Dollins SC, Harris M, Hough AJ Jr. Effect of alkyltins on rabbit articular and growth-plate chondrocytes in monolayer culture. J Toxicol Environ Health 1985;16:229–242.
34. Yamaguchi M, Sugii K, Okada S. Inhibition of collagen synthesis in the femur of rats orally administered stannous chloride. J Pharm Dyn 1982;5:388–393.

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

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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.
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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

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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 3000g, 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^{TM}-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

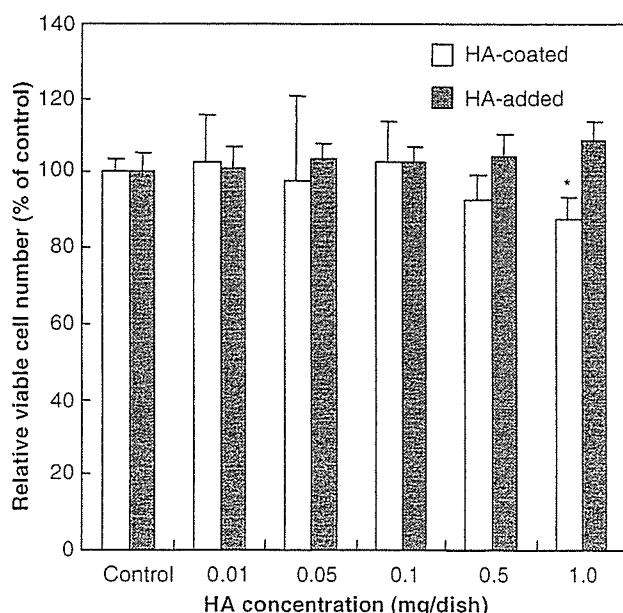

Fig. 1. Concentration-dependent effects of HA-treatment on viability of HIT-T15 cell. After HIT-T15 cells were incubated with HA-coated or HA-added for 24 h, the viable cell numbers of HIT-T15 cell were determined by WST-8 assay as described in methods. Each value denotes the mean ± S.D. of three separate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

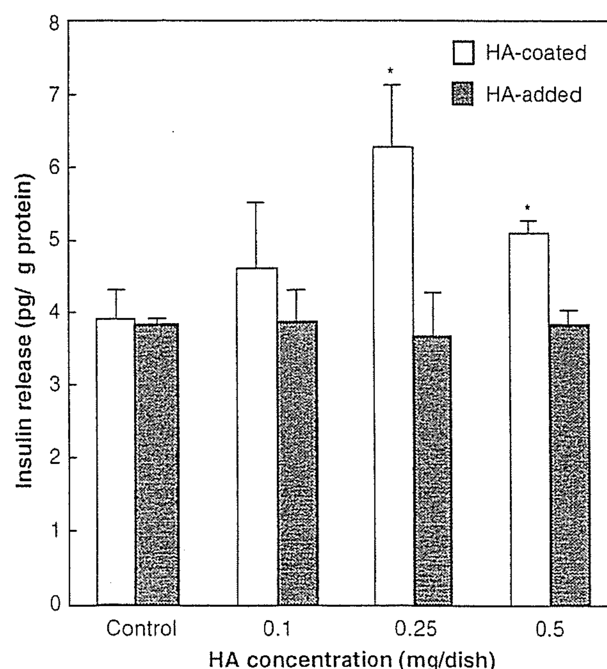

Fig. 2. Insulin secretion from HIT-T15 cells by HA-treatment. HIT-T15 cells were incubated with HA-coating (□) or HA-added (■) for 24 h and then stimulated for 60 min with 11.1 mM glucose in KRB buffer. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the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

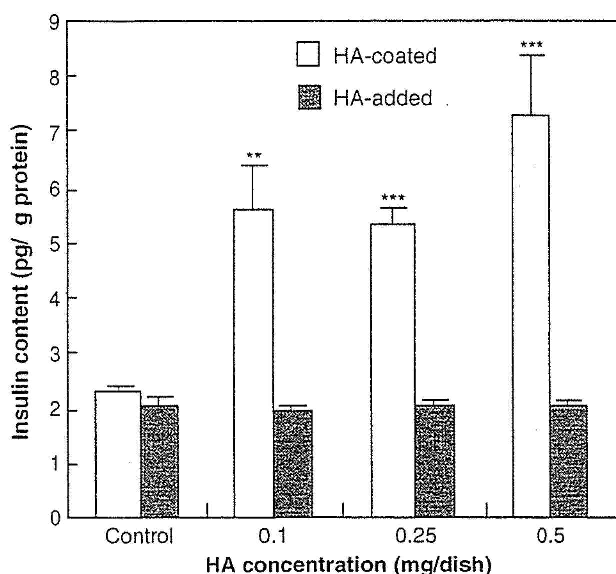

Fig. 3. Insulin content of HIT-T15 cells incubated with HA-coated (□) and HA-added (■). Cells were incubated in the presence of different HA concentrations (0.1–0.5 mg/dish) for 24 h and then stimulated for 60 min with 11.1 mM glucose. The insulin content in the extracts was determined by ELISA insulin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

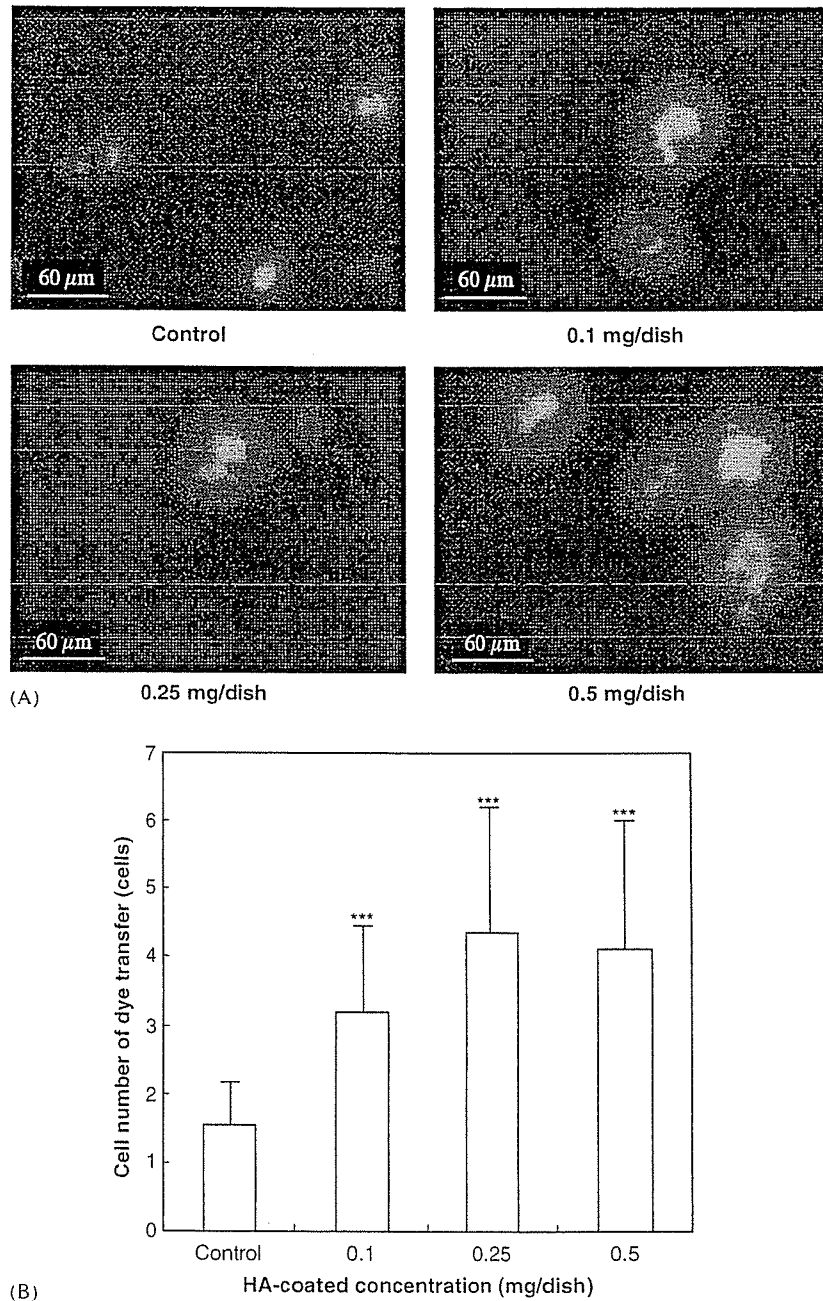

Fig. 4. Concentration-dependent effects of HA-coating on dye transfer in HIT-T15 cells. Cell adherent to glass coverslips were microinjected with 4% Lucifer yellow. Transfer of dye to neighboring cells was assessed by epifluorescence microscopy 2 min later. This is a representative expression of 18 injections 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

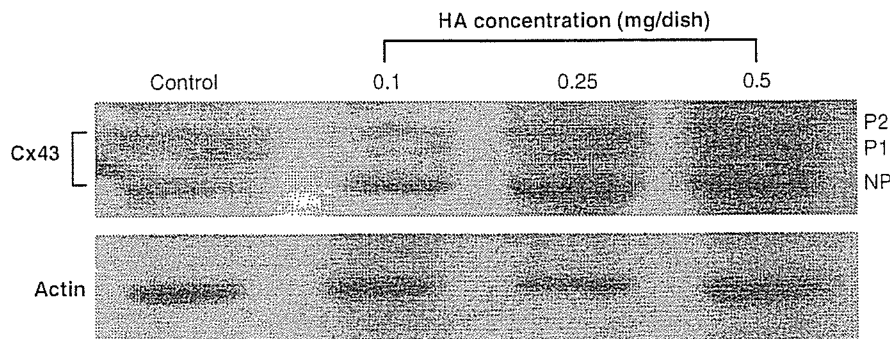

Fig. 5. Identification of Cx43 in HIT-T15 cells grown on the HA-coating dish by Western blot analysis. After HIT-T15 cells were incubated into HA-coated dish for 24 h, cells were lysed and proteins (20 μg) were separated by SDS–PAGE followed by Western blotting using rabbit anti-Cx43 antibody. Actin 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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References

[1] Soon-Shiong P, Heintz R, Yao Q, Yao Z, Zheng T, Murphy M, et al. Insulin independence in a type I diabetic patient after encapsulated islet transplantation. Lancet 1994;343:950–1.
[2] Maki T, Monaco AP, Mullon CJP, Solomon BA. Early treatment of diabetes with porcine islets in a bioartificial pancreas. Tissue Eng 1996;2:299–306.
[3] Laurent TC, Fraser JR. Hyaluronan. FASEB J 1992;6(7):2397–404.
[4] Knudson CB, Knudson W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. FASEB J 1993;7(13):1233–41.
[5] Nagy JI, Hossain MZ, Lynn BD, Curpen GE, Yang S, Turley EA. Increased connexin-43 and gap junctional communication correlates with altered phenotypic characteristics of cells overexpressing the receptor for hyaluronic acid-mediated motility. Cell Growth Differ 1996;7(6):745–51.
[6] Park JU, Tsuchiya T. Increase in gap-junctional intercellular communications (GJIC) of normal human dermal fibroblasts

(NHDF) on surfaces coated with high-molecular-weight hyaluronic acid (HMW HA). Inc J Biomed Mater Res 2002;60(4):541–7.
[7] Meda P. The role of gap junction membrane channels in secretion and hormonal action. J Bioenergy Biomembr 1996;28(4): 369–77.
[8] Meda P, Bosco D, Chanson M, Giordano E, Vallar L, Wollheim C, et al. Rapid and reversible secretion changes during uncoupling of rat insulin-producing cells. J Clin Invest 1990;86(3): 759–68.
[9] Vozzi C, Ullrich S, Charollais A, Philippe J, Qeci L, Medz P. Adequate connexin-mediated coupling is required for proper insulin production. J Cell Biol 1995;131(6 Part 1):1561–72.
[10] Hubbell JA. Materials as morphogenetic guides in tissue engineering. Curr Opin Biotechnol 2003;14(5):551–8.
[11] Park JU, Tsuchiya T. Increase in gap junctional intercellular communications by high molecular weight hyaluronic acid associated with fibroblast growth factor 2 and keratinocyte growth factor production in normal human dermal fibroblasts. Tissue Eng 2002;8(3):419–27.
[12] Nakamura K, Yokohama S, Yoneda M, Okamoto S, Tamaki Y, Ito T, et al. High, but not low, molecular weight hyaluronan prevents T-cell-mediated liver injury by reducing proinflammatory cytokines in mice. J Gastroenterol 2004;39(4):346–54.
[13] Forrester JV, Balazs EA. Inhibition of phagocytosis by high molecular weight hyaluronate. Immunology 1980;40(3):435–46.
[14] Meda P, Chanson M, Pepper M. In vivo modulation of connexin-43 gene expression and junctional coupling of pancreatic β-cells. Exp Cell Res 1991;192(2):469–80.
[15] Charollais A, Gjinovci A, Huarte J, Bauquis J, Nadal A, Martin F, et al. Junctional communication of pancreatic beta cells contributes to control of insulin secretion and glucose tolerance. J Clin Invest 2000;106:235–43.
[16] Meda P, Pepper MS, Traub O. Differential expression of gap junction connexins in endocrine and exocrine glands. Endocrinology 1993; 133(5):2371–8.
[17] Charollais A, Serre V, Mock C, Cogne F, Bosco D, Meda P. Loss of α_1 connexin does not alter the prenatal differentiation of pancreatic β-cells and leads to the identification of another islet cell connexin. Dev Genet 1999;24(1–2):13–26.
[18] Barton W, Cristina A, Isabelle B, Melissa KL, Christopher JR. Glucose-induced translational control of proinsulin biosynthesis is proportional to preproinsulin mRNA levels in islet β-cells but not regulated via a positive feedback of secreted insulin. J Biol Chem 2003;278(43):42080–90.
[19] Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908–10.

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 $1.0 \sim 5.0 \times 10^5$ 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.

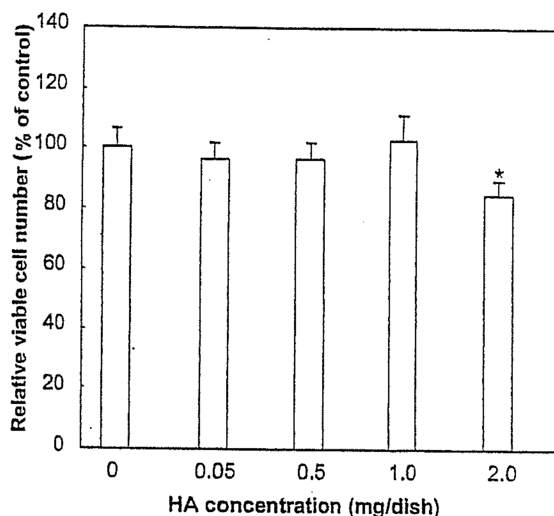

Figure 1. Viability of HIT-T15 cell after 72 h of HA-treatment. The viable cell numbers of HIT-T15 cell were determined by alamarBlue™ assay as described in Section 2. Each value denotes the mean ± S.D. $*P \leq 0.05$ compared to untreated control.

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.

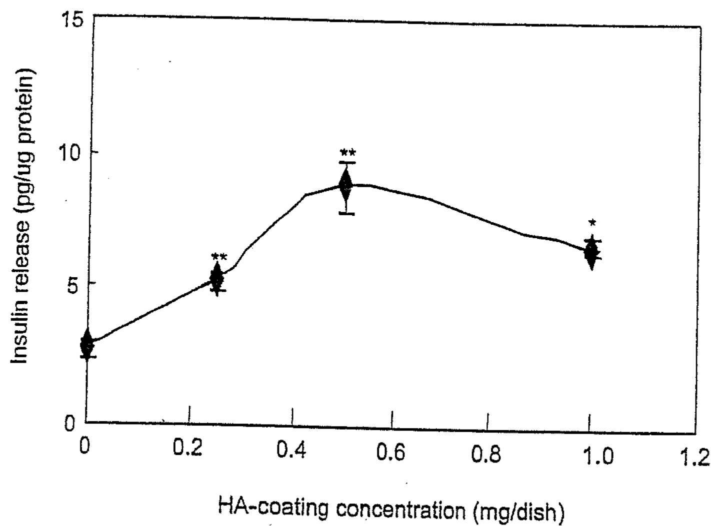

Figure 2. Concentration-dependent effects of HA-coating on insulin secretion from HIT-T15 cells. Treated with HA for 72 h, HIT-T15 cells were incubated with KRB buffer for 60 min. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the mean ± S.D. of three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

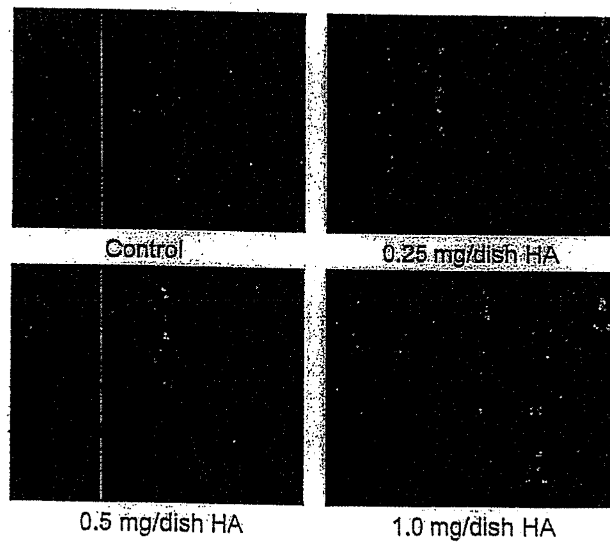

Figure 3. Time-course effects of various concentrations of HA-coating on the dye transfer ratio of HIT-T15 cells.

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.

5. REFERENCES

1. Meda P., Bosco D., Chanson M., Giordano E., Vallar L., Wollheim C. and Orci L. (1990) Rapid and reversible secretion changes during uncoupling of rat insulin-producing cells. *J Clin Invest* 86:759-768

2. Vozzi C., Ullrich S., Charollais A., Philippe J., Qeci L. and Medz P. (1995) Adequate connexin-mediated coupling is required for proper insulin production. *J Cell Biol* 131: 1561-1572
3. Park JU. and Tsuchiya T. (2002) Increase in gap-junctional intercellular communications (GJIC) of normal human dermal fibroblasts (NHDF) on surfaces coated with high-molecular-weight hyaluronic acid (HMW HA). *Inc J Biomed Mater Res* 60: 541-547
4. Nagahata M., Tsuchiya T., Ishiguro T., Matsuda N., Nakatsuchi Y., Teramoto A., Hachimori A. and Abe K. (2004) Anovel function of N-cadherin and Connexin 43: marked enhancement of alkaline phosphatase activity in rat calvarial osteoblast exposed to sulfated hyaluronan. *Biochem Biphys Res Commun* 315: 603-611
5. Meda P. (1996) The role of gap junction membrane channels in secretion and hormonal action. *J Bioenery Biomembr* 28: 369-377
6. Charollais A., Gjinovci A., Huarte J., Bauquis J., Nadal A., Martin F., Andreu E., Sanchez-Andres JV., Calabrese A., Bosco D., Soria B.,m B., Herrera PL. and Meda P. (2000) Junctional communication of pancreatic beta cells contribetes to control of insulin secretion and glucose tolerance. *J Clin Invest* 106: 235-243
7. Cakabrese A., Zhang M., Serre-Beinier V., Caton D., Mas C., Satin SL. and Meda P. (2003) Connexin 36 controls synchronization of Ca^{2+} oscillations and insulin secretion in MIN6 cells. *Diabetes* 52: 417-424
8. Meda P., Chanson M. and Pepper M. (1991) *In vivo* modulation of connexin-43 gene expression an djunctional coupling of pancreaticc β-cells. *Exp Cell Res* 192: 469-480
9. Meda P., Pepper MS. and Traub O. (1993) Differential expression of gap junction connexins in endocrine and exocrine glands. *Endocrinology* 133: 2371-2378
10. Charollais A., Serre V., Mock C., Cogne F., Bosco D. and Meda P. (1999) Loss of α_1 connexin does not alter the prenatal differentiation of pancreatic β-cells and leads to the identification of another islet cell connexin. *Dev Genetics* 24: 13-26

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

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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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1. INTRODUCTION

Brain and neural clinical hospitality have been rapidly advancing, including implantation techniques. Otherwise discreditable accidents sometimes happened. It is necessary to study efficiency and safety of techniques and materials for brain and neural cell proliferation and development. Precise mechanisms by which neurogenesis and gliogenesis are regulated in the central nervous system (CNS) remain to be elucidated. Telencephalic neuroepithelial cells contain neural precursors that give rise to the neuronal lineage and the glial lineage, which includes astrocytes and oliogodendrocytes (1, 2). The fate of neural precursors in the developing brain is believed to be determined by intrinsic cellular programs and by external cues, including implantation of biomaterials and cytokines (3). Doetsch et al. demonstrated that subventricular zone (SVZ) astrocytes act as neural stem cells in both the normal and regenerating brain (4). Neural stem cells, endogenously present in spinal cord *in vivo*, proliferate in response to injury, yet the vast majority of newly generate cells are glial fibrillary acidic protein (GFAP)-positive astrocytes (5). In addition, adult hippocampus-derived neural stem cells, when implanted into adult brain in such a region as cerebellum or striatum, have been reported to differentiate predominantly into glial cells (2, 6, 7).

Biodegradable polymers have been attractive candidates for scaffolding materials because they degrade and the new tissues are formed, although adverse events such as foreign-body reaction, inflammation and tumor formation were reported in clinical human and animal study. These scaffolds have shown great promise in the research of engineering a variety of tissues. Biodegradable polymer poly (L-lactic acid) (PLLA) is frequently implanted in cranial surgery etc. However, to engineer clinically useful tissues and organs is still a challenge. The understanding of the principles of scaffolding is far from satisfactory, still more its effect and safety on neural tissues are not known. We previously reported PLLA suppressed proliferation and differentiation of fetal rat midbrain neural precursor cells (8). In this report, we investigated the effect of PLLA on normal human astrocytes (NHA).

2. MATERIALS AND METHODS

Astrocyte cell culture

We used normal human astrocyte (Cambrex Bio Science, Walkersville, MD). NHA were seeded into 12-well plates for quantitative RT-PCR at a density of 2×10^4/well, or 24-well plates for MTT assay at a density of 1×10^4/well in ABM medium(Cambrex Bio

Science) supplemented with 5% FCS, rhEGF and IGF, and cultured in a humidified atmosphere of 5% CO_2 in 95% air at 37°C.

PLLA preparation

Stock solutions of PLLA were made in dimethyl sulfoxide (DMSO) and final concentration of DMSO was 0.1%; this concentration did not affect proliferation and development of NHA. Control cultures were incubated with 0.1% DMSO. Stock solutions of lactic acid and tin chloride were made directly in ABM medium.

MTT assay

After cell culturing for 1 week with PLLA, the viability of NHA cells was determined by MTT assay. The TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan) was used to measure changes of cell numbers. This assay is a nonradioactive alternative to tritium-thymidine incorporation. The system measures the conversion of tetrazolium salt compound into a soluble formazan product by the mitochondria of living cells. NHA in 24-well plates were cultured as described above. One week after NHA cultured with vehicle or PLLA, the media were replaced with 300 μl of fresh medium containing 6 μl TetraColor ONE reagent. After 2h, samples were measured in a micro plate reader.

Expression of neural cell marker genes

Total RNA was prepared from NHA using a modified acid guanidium thiocyanate-phenol-chloroform method. The total RNA treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) were subjected to reverse transcription using oligo d(T) primer (Toyobo, Tokyo, Japan) and superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42°C for 30 min followed by RNase H treatment. Aliquots of the cDNA (1/20) were used as templates for PCR analysis using Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 μl mixture including 1 μl of RT reaction, 2 μl Light Cycler-Fast Start Reaction Mix SYBR Green 1(Roche, Mannheim, Germany), 0.5 μM/liter of each primer, and 3 mmol/liter $MgCl_2$. The PCR program consisted of 40 cycles of 8 sec at 94°C, 5 sec at 65°C, 10 sec at 72°C. Primer sequences for amplification are 5'- CTAAGGAGGAGATTGGACAGG-3' and 5'-AGTGGTGGCAGTGATTT CAGT-3' for Nurr-1 amplification, 5'-TCCGCTGCTCGCCGCTCCTAC-3'and 5'-TCATCTCTGCCCGCTCACTGG -3' for GFAP amplification, 5'-TCGCCCTGCCCACTTGACTTC-3' and 5'-TTCCACACCTCCACGCTC TGA-3' for Id-3 amplification, 5'-GAGATCAGAGCCCAGGATGCT-3' and 5'-CTGAGGGGTGGTGCCAAGGAG -3' for Nestin amplification, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-

TCCACCACCCTGTTGCTGT A-3' for GAPDH. RNA preparation and RT-PCR in the present study were performed in triplicate.

Statistical analysis

The Fisher's PLSD was used to compare the PLLA concentration and relative expression levels of neural specific marker mRNA.

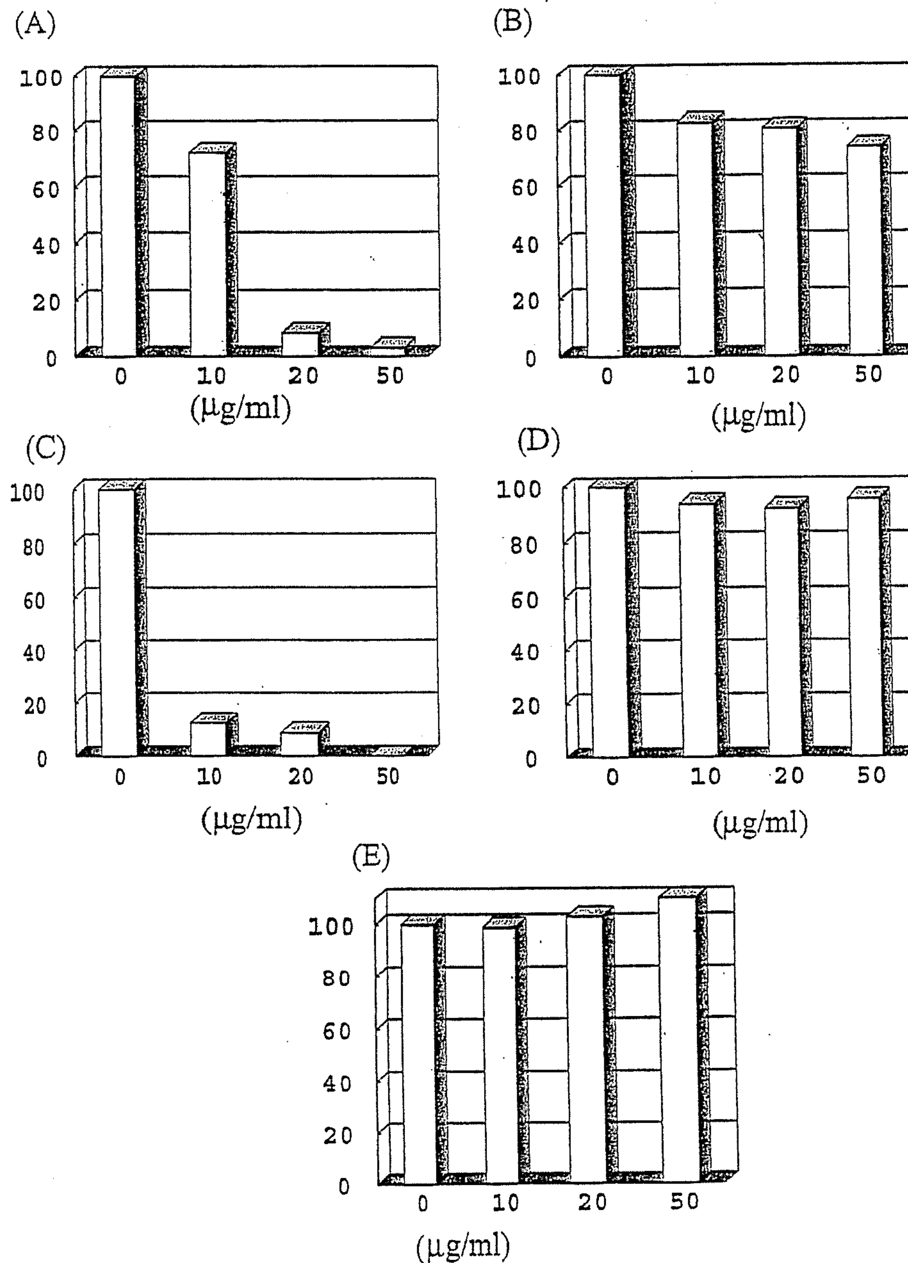

Fig. 1 Effect of PLLA on NHA proliferation (A) PLLA 3.000 (B) PLLA 5.000 (C) PLLA 11.000 (D) Lactic acid (E) Tin chloride

3. RESULTS AND DISCUSSION

NHA proliferation

We used three kinds of PLLA. PLLA 3000 (PLLA, Mw 3000) is made without catalyst. PLLA 6000 (PLLA, Mw 5000) is made with organic tin catalyst. PLLA 11000 (PLLA, Mw 11000) is made with catalyst tin chloride, contains 590 ppm tin. After a week culture with PLLA, we detected cell number of NHA using MTT assay. Cell numbers were decreased in a dose-dependent manner of PLLA (Fig. 1A-C). The cell number of NHA cultured with 50 μg/ml of PLLA 3000, PLLA 5000 and PLLA 11000 were 15%, 70% and 7.8% of that of control respectively.

Whether tin ion included in PLLA affected NHA proliferation or not, we added tin chloride to NHA culture medium (Fig. 1D). The concentration of tin chloride at 50 ng/ml did not affect NHA proliferation. PLLA is hydrolysed in medium, we assayed lactic acid (LA), a monomer of PLLA was also tested by the MTT assay using NHA cells. (Fig. 1E). There was no effect on the cell number of NHA culture with LA monomer. The cause of PLLA effect for NHA was neither included tin ion nor degraded LA monomer. It was probably the effect of PLLA itself and/or degraded LA oligomers.

Lam and his co-workers demonstrated that predegraded PLLA (P-PLLA; 25 kGy gamma-irradiation) caused signs of cell damage, cell death, and cell lysis due to phagocytosis of a large amount of P-PLLA particles (9). Phagocytosis of LA oligomers or degraded PLLA particles may affect the proliferation and development of NHA. It is necessary to know culture medium with PLLA contains how much PLLA particles, PLLA oligomer and organic tin.

Gene expression of neural cell specific markers

It has been suggested that a part of astrocytes contain neural precursor cell activity that give rise to neuron, oligodendrocyte and astrocyte itself. The recent discovery of stem cell populations in the CNS has generated intense interest, since the brain has long been regarded as incapable of regeneration (5, 10, 11). Neural stem cells (NSCs) have capability for expansion and differentiation into astrocytes, oligodendrocytes, and neurons in vitro (12, 13). NSCs have been suggested to have therapeutic potential for central nervous system regeneration (14-16).

They express their original specific genes, neural cell specific markers. Neural precursor cells express Nestin, a class IV intermediate filament protein. Differentiated neuron expresses Nurr-1, a transcription factor and Id-3, a transcription inhibitory factor. Astrocyte expresses