

diameter [5,9]. Taking into account our findings about the inhibitory effects of various microspheres on the functions of NHOst, it is probable that NHOst can phagocyte PS microspheres as well as macrophages, and in particular, may phagocyte microspheres 5 μm in diameter. Moreover, the effect of the added PS microspheres suggests that NHOst better recognize the microspheres from their lower than upper side. This may explain the reduced functions of NHOst co-cultured with the pre-coated 5 μm PS microspheres.

To estimate the effect of the material composing the microspheres, NHOst were cultured for 1 week on pre-coated PS, PE, alumina and HAp microspheres, all of which have a diameter of around 5 μm . Table 1 shows their number ratio and ALP activities, and the calcium amounts. Pre-coated PS, PE and alumina microspheres showed the potential to suppress functions of NHOst although some of these data did not show statistical differences against NHOst without microspheres. However, when NHOst were cultured with pre-coated HAp, the amount of calcium deposited was almost twice that detected in the cells without microspheres. It was observed that HAp microspheres have no potential to deposit calcium after a 1-week incubation without NHOst (data not shown). Therefore, the increase in calcium deposition by pre-coated HAp may be due to the enhancement in the differentiation of NHOst in contact with HAp. As expected, added various microspheres affected NHOst in a similar manner but less than the pre-coated microspheres (data not shown). We have hypothesized that GJIC of cells in contact with various biomaterials can be used as an index for estimating the biocompatibility of many kinds of biomaterials [5,6,9,11]. In addition, osteoblasts have been reported to communicate with one another *via* GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development [8,12]. Therefore, effects of these microspheres on the communication of co-cultured NHOst were estimated to consider the relation between this function and the differentiation of NHOst. The FRAP assay revealed that HAp microspheres enhanced the GJIC level of NHOst to 1.8 times as much as that of NHOst alone but others slightly inhibited it, indicating HAp has a potential to enhance homeostasis maintenance function of the NHOst as well as their differentiation. Details of the microspheres effects on GJIC of NHOst will be reported elsewhere [13]. These results indicated that the materials of microspheres affected the differentiation of co-cultured NHOst as well as the diameter of microspheres and their contact with the cells. In addition, microspheres made from HAp, which is a major component of bone tissue and has been shown to have good biocompatibility as bone substitute implants [14], may have the potential to enhance the differentiation of osteoblasts. These results suggest that the estimation of the effects of biomaterials in microsphere form on in vitro cell function may be useful for their in vivo biocompatibility evaluation.

We estimated the effect of sintering, normally used to harden HAp, on the function of NHOst. The estimation revealed that both HAp microspheres enhanced the amount of calcium deposited although the ALP activity of the cells decreased. In addition, when the un-sintered HAp microspheres were incubated with NHOst, the calcium deposition was observed more than sintered HAp. As another index of the differentiation of the NHOst, mRNA expression levels of osteocalcin, which is a well-known protein detected in

Table 1. Effects of a 1-week incubation with pre-coated microspheres on various functions of NHOst.
(Amounts of microspheres = 20 μg /well)

	Control	Polystyrene	Polyethylene	Alumina	Hydroxy Apatite (Sintered)
Diameter (μm)		5.0	6.4	5.1	7.2
The cell number ratio (%)	100.0 \pm 5.5	88.2 \pm 2.2	92.2 \pm 1.3	82.4 \pm 2.8	83.0 \pm 2.3
Percent ALP activity (activity/proliferation)	100.0 \pm 4.7	79.2 \pm 5.6	72.7 \pm 3.6*	58.2 \pm 5.7*	73.8 \pm 6.0*
Percent deposited calcium (Calcium percent/proliferation)	100.0 \pm 3.7	97.3 \pm 4.2	82.3 \pm 3.7	90.3 \pm 7.8	163.3 \pm 18.5*(a)

Data are shown as the mean value \pm SEM (n = 4 to 22)

* p < 0.01, against control group

(a) p < 0.05, against NHOst co-cultured with polyethylene and alumina microspheres

differentiated osteoblasts [15], were determined using the RT-PCR technique. Figure 2 shows time profiles of osteocalcin mRNA expression in NHOst cultured with pre-coated PS, PE, alumina, and two kinds of HAp microspheres. As shown in the figure, only the cells co-cultured with sintered HAp microspheres expressed osteocalcin mRNA after a 1-day incubation, while those co-cultured with other microspheres did not express the mRNA. This finding suggests that sintered HAp microspheres have the potential to induce osteocalcin production from NHOst. Neither spontaneous calcium deposition was observed by the incubation of sintered nor un-sintered HAp microspheres without NHOst, so that it is possible that the un-sintered HAp degrade in culture medium with NHOst, resulting in an increase of calcium concentration in the culture medium that enhances the calcium deposition by the NHOst. Therefore, it is suggested that sintered HAp can induce the differentiation of NHOst, and may be a suitable material for inducing osteogenesis rather than un-sintered one.

In conclusion, microspheres made from various materials had an effect on the differentiation of NHOst. The level of the effect varied with the size, amount, and composition of the microspheres. Microspheres made from PS, PE and alumina showed a potential to suppress the proliferation and the differentiation of co-cultured NHOst. On the other hand, microspheres made from HAp, especially sintered HAp, enhanced the differentiation of co-cultured NHOst, and showed their potential to maintain their homeostasis. Estimating the effect of various microspheres on the differentiation of osteoblasts will provide valuable information on the effects of wear debris from artificial hip joints as well as estimating their effects on osteoclast function.

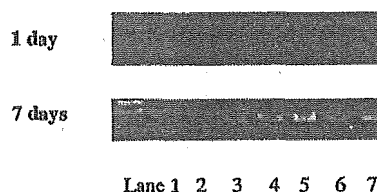


Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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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 μ g/

ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. The subculture cells were seeded at a density of 1.0–5.0 \times 10⁵ 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 \times 10⁵) 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 \times 10³) 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 Ca²⁺/Mg²⁺, 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 Ca²⁺/Mg²⁺-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 μ g protein/lane) were fractionated by electrophoresis 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 (≤ 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 (≥ 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 (≤ 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

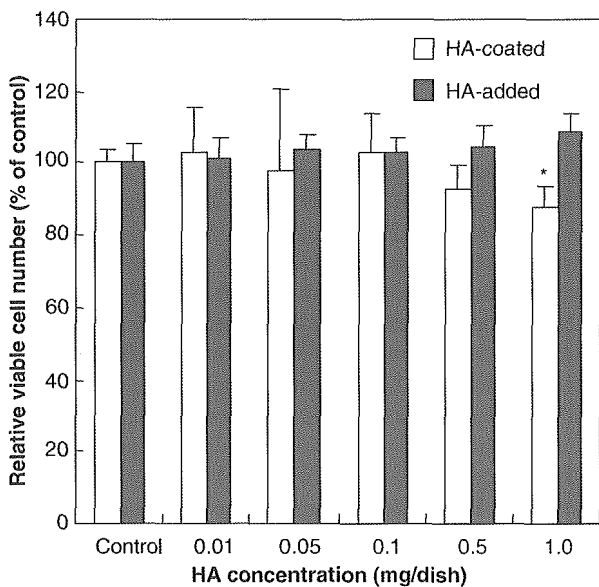


Fig. 1. Concentration-dependent effects of HA-treatment on viability of HIT-T15 cell. After HIT-T15 cells were incubated with HA-coated or HA-added for 24 h, the viable cell numbers of HIT-T15 cell were determined by WST-8 assay as described in methods. Each value denotes the mean \pm S.D. of three separate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

and 3.90 ± 0.41 pg/ μ g 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

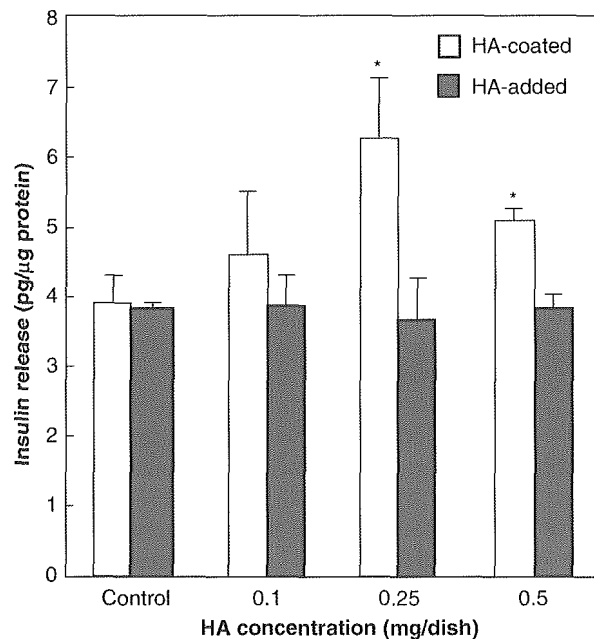


Fig. 2. Insulin secretion from HIT-T15 cells by HA-treatment. HIT-T15 cells were incubated with HA-coating (\square) or HA-added (\blacksquare) for 24 h and then stimulated for 60 min with 11.1 mM glucose in KRB buffer. The released insulin in the spent medium was determined by ELISA insulin kit. Each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

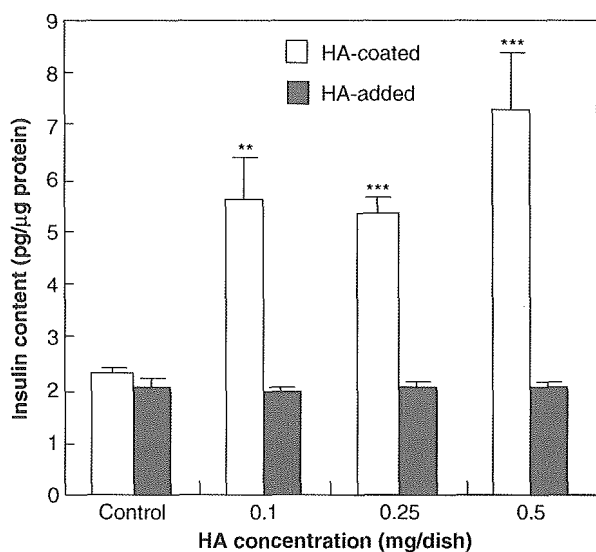


Fig. 3. Insulin content of HIT-T15 cells incubated with HA-coated (□) and HA-added (■). Cells were incubated in the presence of different HA concentrations (0.1–0.5 mg/dish) for 24 h and then stimulated for 60 min with 11.1 mM glucose. The insulin content in the extracts was determined by ELISA insulin kit. Each value denotes the mean \pm 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

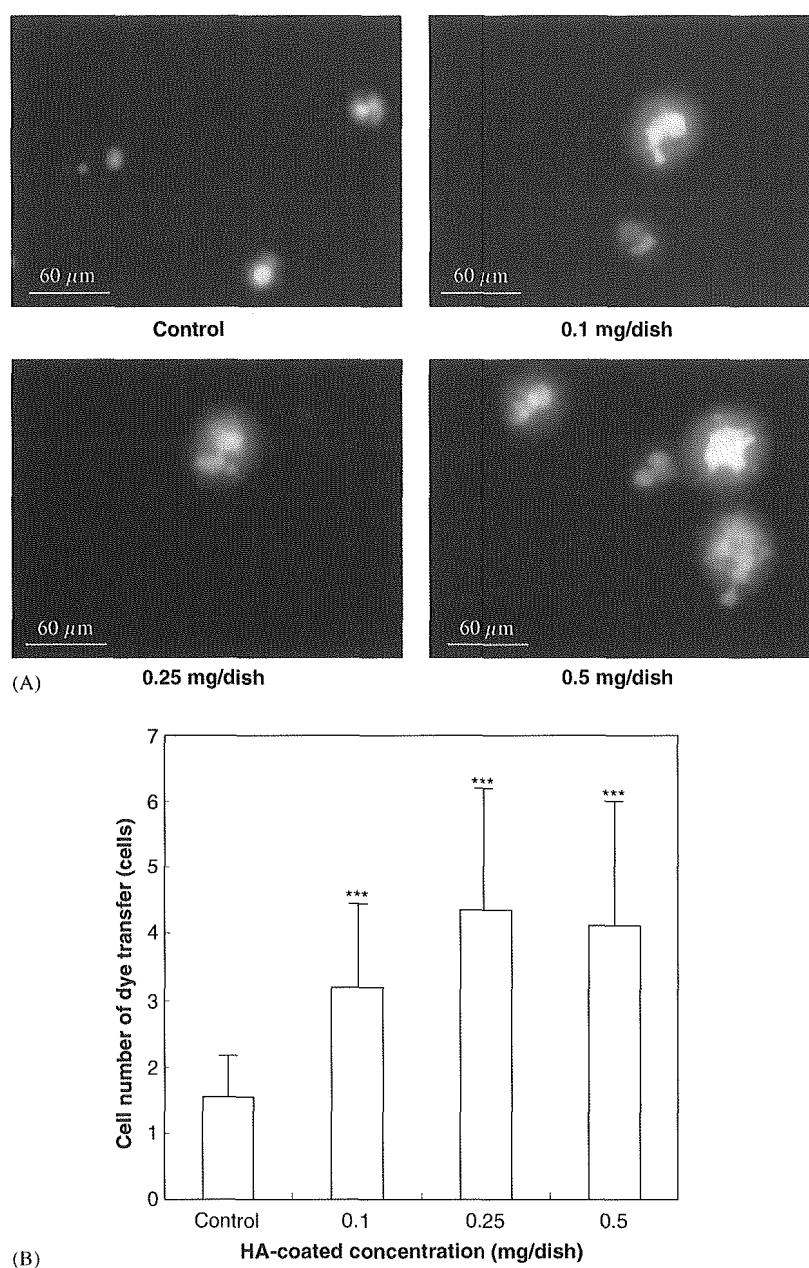


Fig. 4. Concentration-dependent effects of HA-coating on dye transfer in HIT-T15 cells. Cell adherent to glass coverslips were microinjected with 4% Lucifer yellow. Transfer of dye to neighboring cells was assessed by epifluorescence microscopy 2 min later. This is a representative expression of 18 injections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

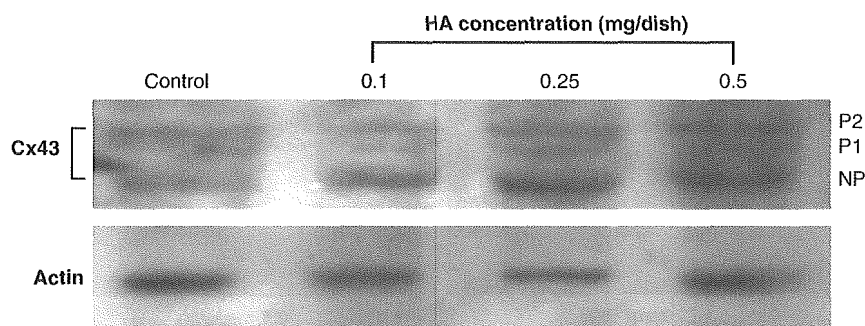


Fig. 5. Identification of Cx43 in HIT-T15 cells grown on the HA-coating dish by Western blot analysis. After HIT-T15 cells were incubated into HA-coated dish for 24 h, cells were lysed and proteins (20 μ g) were separated by SDS-PAGE followed by Western blotting using rabbit anti-Cx43 antibody. Actin 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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A NOVEL NON-DESTRUCTIVE METHOD TO MEASURE ELASTIC MODULI OF CARTILAGE CELL IN SITU

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Abstract: In order to determine the right occasion to transplant the cultivated cellular tissues to the diseased part, it is important to grasp their mechanical properties as one of the evaluation method of the degree of ripeness in the regenerative medicine for the cartilage and bone. However, the mechanical property measurement for the minute soft tissues has been almost impossible in the conventional methods to measure their stresses and/or strains, due to their difficulty of formability into specimens. Thereupon, we developed a novel method that enables to measure the bulk modulus of elasticity (modulus of elasticity of volume) by using the relation between volume and pressure of the soft living tissues in situ. Some results of the measurements of the cartilage cell tissues in the cultivation in different materials' scaffolds, different medias for several weeks were obtained. The results of soft silicone rubber were compared with those obtained by the conventional biomechanical measurement, and confirmed its accuracy. It was found that this novel method has been effective to measure the volume change of the minute living soft tissues precisely. This method could evaluate the strength of the cell tissues during cultivation continually, because it is able to measure in a short time without damaging the specimen.

Introduction

In order to determine the right occasion to transplant the cultivated cellular tissues to the diseased part, it is important to grasp their mechanical properties as one of the evaluation method of the degree of ripeness in the regenerative medicine for the cartilage, ligament and bone.

Many biomechanical experimental approaches have been done to measure the mechanical properties of soft tissues include an artificial cartilage. In addition, the classical compression tests, utilizing unconfined tension, compression, indentation, and confined compression on full-thickness cartilage or soft tissues samples have been

invaluable tools to determine more exact material properties in biomedical engineering fields.

However, it is very difficult in conventional methods to measure the biomechanical properties of soft tissues such as skeletal muscles and cartilages. This is not only due to the technical difficulties associated with invasive measurements but also because geometries of living tissue results from irregular forms.

Therefore, in this study we developed a novel method that enables to measure the bulk modulus of elasticity (modulus of elasticity of volume) by using the relation between volume and pressure of the soft living tissues in situ.

Materials and Methods

Theory and Experimental device

Figure 1 shows the diagrammatic illustration of our device, which are used in a pressure pot under different air pressures. Chamber 1 is for specimen and chamber 2 is constructed for references in our device.

A sensor detects the difference of pressure change (ΔP) produced when the change is given. In order to calculate the relationship between the volume (V) and the pressure (P), we used the classical equation given by

$$\Delta P_1 - \Delta P_2 = \frac{nP_1\Delta V_1}{V_1} - \frac{nP_2\Delta V_2}{V_2} \quad (1)$$

where P_1 , V_1 are, respectively, the pressure and volume in chamber 1, and P_2 , V_2 are the pressure and volume in chamber 2. n is the number of moles. If the volume change (V_x) is occurred in chamber 1 for the specimen, the equation (1) can be rewritten by

$$\Delta P_1 - \Delta P_2 = \frac{nP_1\Delta V_1}{V_1 - V_x} - \frac{nP_2\Delta V_2}{V_2} \quad (2)$$

Here, when the pressure increased, ΔP is inserted into equation (2).

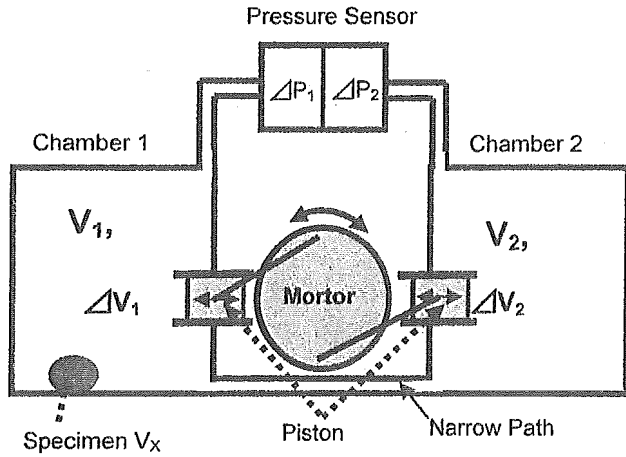


Fig. 1 Schematic diagram of our device (P_1 , V_1 are the pressure and volum in chamber 1, and P_2 , V_2 are the pressure and volum in chamber 2. n is the number of moles. V_x is the volume change of specimen.)

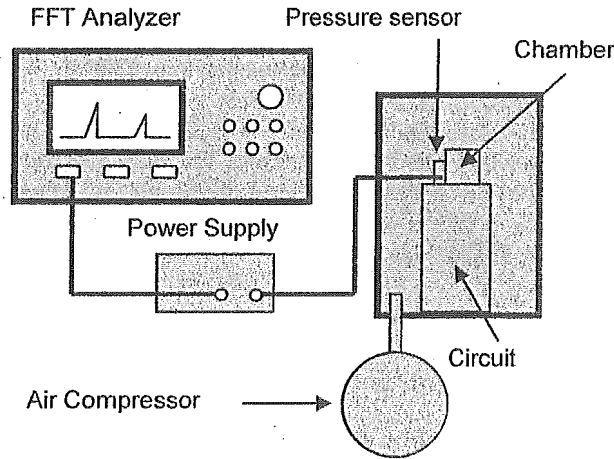


Fig. 2 The composition outline of the measurement equipment for the calculating the mechanical property of soft tissues based on the pressure-volume relationship

$$\Delta P_1 - \Delta P_2 = \frac{n(P + \Delta P)\Delta V}{V_1 - V_x} - \frac{n(P + \Delta P)\Delta V}{V_2} \quad (3)$$

$$= n(P + \Delta P)\Delta V \left(\frac{1}{V_1 - V_x} - \frac{1}{V_2} \right)$$

Figure 2 shows the composition outline of the measurement equipment. The bulk modulus calculated by following equation (4).

$$K = P / \epsilon_v \quad (4)$$

where K , P and ϵ_v is bulk modulus, pressure and volum strain.

Table 1 Volume and the physical-properties value of the steel chosen as quality of a standard, and a silicone rubber sample

Specimen Type	Volume (V , mm^3)	Young's Modulus (E , kPa)	Bulk Modulus (K , kPa)
Steel Ball	4.19	200×10^6	170×10^6
Silicon Rubber	11.5	50	417*

* : This value is calculated with $\nu = 0.48$

Table 2 The contents of samples on cartilages

Sample	Content	Scaffold	
Cartilage	DMEM (+)	Within using cartilage cell	Collagen gel
	DMEM (-)	Without using cartilage cell	

Specimen preparation

The silicon rubber and steel ball, which having the known mechanical properties, were used in the validation of our specially designed device. The elasticity silicone rubber which a dynamic physical-properties value is known and approximates to it of soft tissues as the quality of a standard of the measurement for a living body organization was chosen in this study. In Table 1, the known physical-properties values were shown with the physical-properties value of a steel ball.

We measured the bulk modulus of cartilage cells with using our newly designed device. The human cartilage cells cultivated for four weeks in a scaffold and DMEM culture solution of collagen gel was measured as a sample. In this measurement, two types of cartilage cell with (+) and without (-) using DMEM The content of samples are shown in Table.2.

Results and Discussion

Figure 3 shows the volume changes of the silicon rubber in relation to the changed of pressure. The volume decreased with increasing pressure, respectively. The bulk modulus of two pieces of silicone rubber calculated from equations of (3) and (4) were an average of 409.1kPa. The bulk-modulus of 417kPa obtained from the conventional mechanical device was compared to the value calculated by our device as shown in Fig. 4. Only 2% error was produced, and it can be understood as the exact value having been acquired.

In this study, we measured the bulk modulus of a cartilage cell tissue cultivated in DMEM solution on collagen gel. Figure 5 shows the volume changes of the cartilage cell tissues under the pressures.

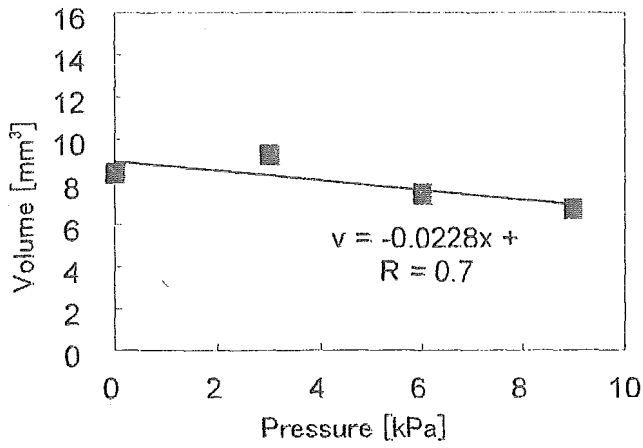


Fig. 3 The volume changes in relation to changes of the pressures on the two silicon rubbers with different mechanical properties

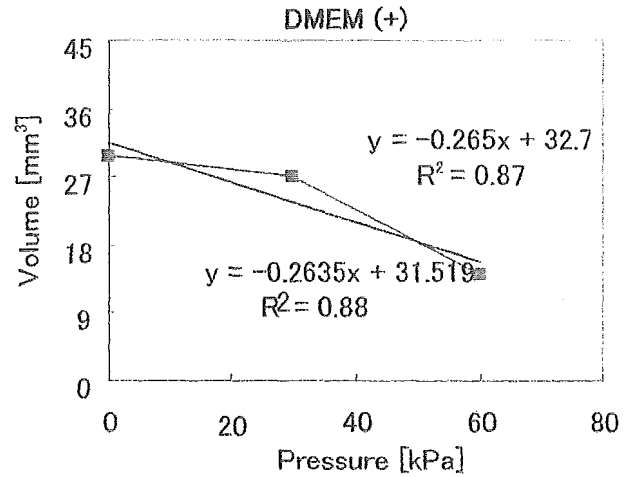


Fig. 5 The volume changes in relation to changes of the pressures on the two types of cartilage within DMEM(+) and without DMEM(-)

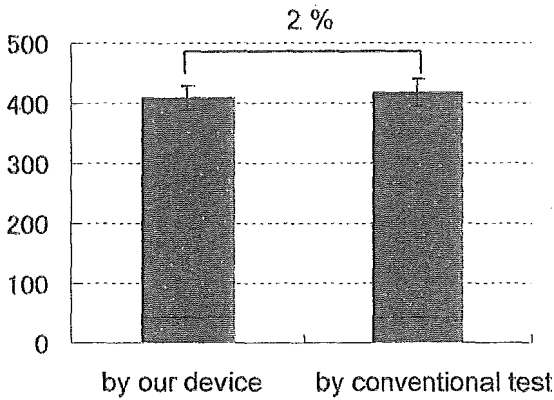


Fig. 4 The Bulk Modulus (K) of silicon rubber with the unknown the mechanical properties

The bulk modulus of cartilage cell tissue were about 120kPa and 90kPa corresponding to the each DMEM(+) and DMEM(-). However, it should be carefully investigated that the bulk modulus of the cartilage cell tissue calculated by our device will be exact or not with the other biomechanical method.

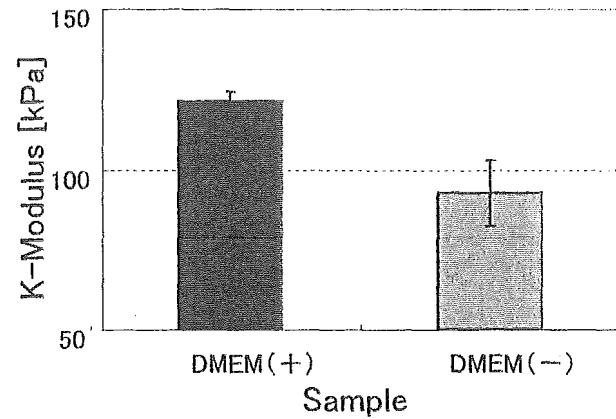
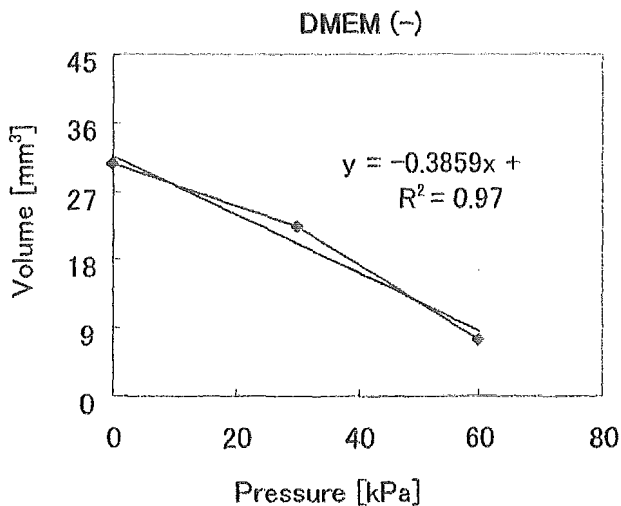


Fig. 6 The bulk moduli of the cartilage cell tissues in a collagen scaffold after four week cultivation within DMEM (left) and without DMEM (right).

Conclusions

It was found that this novel method has been effective to measure the volume change of the minute living soft tissues precisely. This method could evaluate the strength of the cell tissues during cultivation continually, because it is able to measure in a short time without damaging the specimen.

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Hydroxy apatite microspheres enhance gap junctional intercellular communication of human osteoblasts composed of connexin 43 and 45

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Abstract: The aseptic loosening of artificial joints with associated periprosthetic bone resorption may be partly due to the suppression of osteoblast function to form new bone by wear debris from the joint. To assess the effect of wear debris on osteoblasts, effects of model wear debris on gap junctional intercellular communication (GJIC) of normal human osteoblasts were estimated. The GJIC activity of the osteoblasts after a 1-day incubation with the microspheres was similar to that of normal osteoblasts. However, hydroxy apatite particles, which have been reported to enhance the differentiation of osteoblasts in contact with them, enhanced the GJIC function of the osteoblasts. From RT-PCR studies, not only connexin 43 but also connexin 45 is suggested to play a role in the GJIC of the osteoblasts in an early stage of

coculture with the microspheres, although it is still unclear how these connexins work and are regulated in the GJIC and differentiation. However, this study suggests that there is a relationship between the early levels of GJIC and the differentiation of the cells. Therefore, estimating the effect of biomaterials, even in the microsphere form, on the GJIC of model cells, with which the biomaterials may be in contact *in vivo*, can provide important information about their biocompatibility. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 74A: 181–186, 2005

Key words: gap junctional intercellular communication; human osteoblasts; microspheres; hydroxy apatite; connexin

INTRODUCTION

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, sometimes resulting in undesirable side effects. One well-known example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. It has already been reported that aseptic loosening with associated periprosthetic bone resorption is partly due to the activation of macrophages and osteoclasts by wear debris from the artificial joint.^{1–14} Macrophages stimulated by wear debris *in vitro* release significant amounts of inflammatory mediators such as interleukin-1, interleukin-6, prostaglandin E2, collagenase, and tumor necrosis factor.^{6–14} In addition, the biological effects of wear debris may depend on the type of material used as well

as the shape, size, and amount of the debris.^{4–11} Therefore, it is important to estimate the biocompatibility of biomaterials with not only their original shape but also possible transformed shapes after their usage.

During the last decade, we have been researching the inhibitory potential of many kinds of biomaterials on gap junctional intercellular communication (GJIC) as an index for their biocompatibility.^{15–18} GJIC is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules, which results in regulating cell growth, development, and differentiation of cells.^{19,20} Therefore, it is reasonable that disruption of this function is the cause of many kinds of diseases. In a previous report,¹⁸ we examined the inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer *in vivo*, on the GJIC of rodent-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in the body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joints by estimating their effect on GJIC function.

No benefit of any kind will be received either directly or indirectly by the authors

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However, it must be noted that the effects of the microspheres may be different when the effects on the GJIC of human-derived cells are estimated. Osteoblasts have been reported to communicate with one another via GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development.^{21,22} Therefore, the question is raised whether wear debris has an inhibitory effect on the GJIC and the GJIC inhibition has a relation with the aseptic loosening of artificial joints. Because we have already observed some precoated polymer microspheres around 5 μm in diameter showed the potential to inhibit GJIC of fibroblasts contacting with them,²³ we estimated effects of various microspheres around 5 μm in diameter on GJIC function using normal human osteoblasts to discuss the relationship between the GJIC and the differentiation of osteoblasts. In this study, we employed fluorescence recovery after photobleaching (FRAP) analysis for estimating the GJIC function,¹⁷ and assessed the potential effect of many kinds of microspheres on the GJIC.

MATERIALS AND METHODS

Microspheres

Monodispersed polystyrene (PS) microspheres (5 μm in diameter) were purchased from Japan Synthetic Rubber Co., Ltd. (Tokyo, Japan). Low-density polyethylene (PE) microspheres were generously supplied by Sumitomo Seika chemicals Co., Ltd. (Tokyo, Japan). Alumina (Al_2O_3) microspheres were obtained from the Association of Powder Process Industry and Engineering. Sintered hydroxyapatite microspheres (HA, 7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. A Multisizer II (Coulter Electronics Inc., Hialeah, FL) was used to determine the average diameter of PE and alumina microspheres: 6.4 and 5.1 μm , respectively. Microspheres were sterilized by dispersing them in a 70% ethanol solution, followed by centrifugation in sterile conditions to remove the ethanol solution. The microspheres were dispersed in sterile methanol for cell differentiation tests at specified concentrations. The suspension of microspheres in methanol was added to 35-mm type I collagen-coated cell culture dishes (Asahi techno glass, Chiba, Japan), and the plates dried overnight at room temperature. The obtained microsphere-coated dishes (100 μg /dish) were subjected to the assays.

Cell culture

Normal human osteoblasts (NHOst) were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo, Japan). The cells were

maintained in incubators under standard conditions (37°C, 5%-CO₂-95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst (1×10^5 cells/dish/2.5 mL medium) were cultured on microsphere-coated dishes for estimating the effect of the microspheres interacted from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst cells were cultured with microsphere-containing medium (100 μg /2.5 mL medium) after they had adhered to the collagen-coated dishes. The test cells were cultured while changing the medium three times when the measurement of GJIC was performed after a 7-day incubation.

Measurement of GJIC activities

NHOst cultured with microspheres were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these microspheres toward the GJIC. FRAP analysis was carried out according to an original procedure by Wade et al.,²⁴ with some modifications.¹⁷ Briefly, NHOst were plated on microsphere-coated dishes and incubated for 1 or 7 days. After a wash with phosphate buffer saline (PBS) containing MgCl_2 and CaCl_2 [PBS(+)], the cells were incubated for 5 min at room temperature in PBS(+) containing 5,6-carboxyfluorescein diacetate (7 $\mu\text{g}/\text{mL}$, excitation 488 nm and emission 515 nm). After the washing off of excess extracellular dye with PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. In the control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure as the tested cells. Cells in contact with test microspheres and at least two other cells were subjected to FRAP analysis under an Ultima-Z confocal microscope (Meridian Instrument, Okemos, MI) with a 10 \times objective lens at room temperature. The cells were photobleached with a 488-nm beam and the recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total period of 4 min. The data obtained from more than seven independent cells were expressed as the average of fluorescence recovery rate in comparison to the rate obtained from NHOst cultured without microspheres.

Effect of microspheres on calcium deposition by NHOst

The amount of calcium deposited during a 7-day incubation of the cells were evaluated as follows: NHOst were cocultured with either precoated or added microspheres in 24-well collagen-coated culture plates (Asahi techno glass Chiba, Japan) for 1 week (2×10^4 cells/20 μg microspheres/well/500 μL medium). After the cells were fixed in formaldehyde, 0.5 mL of 0.1 M HCl was added to each well after washing the cells with PBS. The amounts of calcium dissolved in HCl were estimated using a Calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to the manufacturer's direction.

RT-PCR for estimating expression of connexins

According to the method reported by Ichikawa et al.,²⁵ RT-PCR was performed to detect the expression of connexin mRNA in NHOst. After culturing NHOst with microspheres for a scheduled time, total RNA was extracted from the NHOst using TRIZOL[®] reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. After dissolving the RNA in diethylpyrocarbonate-treated water, the total RNA concentration was measured spectrophotometrically using Genequant (Amarsham Biosciences Corp., Piscataway, NJ). RNA samples were adjusted to a minimum concentration among collected samples in each experiment and reversibly transcribed to cDNA using Superscript[™] II (Invitrogen Corp.). For PCR amplification of human connexin 45, Takara Ex-Taq[™] (Takara Shuzo Co., Ltd., Shiga, Japan) was used with Ex-Taq[™] buffer consisting of 20 pmol each of two human connexin-45 specific primers (forward 5'GTGGCAACTCCCTCTGTGAT3' and reverse 5'GGATCCTCAAGTCCCTCCT3'). For PCR amplification of human connexin 26, 32, and 43, Takara LA-Taq[™] (Takara Shuzo Co., Ltd.) was used with Ex-Taq[™] buffer consisting of 6 pmol each of the human connexin-specific primers (for connexin 26, forward 5'ATGGATTGGGCACGC3' and reverse 5'TTAAACTGGCTTTTTTGACTTCCC3'. For connexin 32, forward 5'ATGAACTGGACAGGTTGTACACCTTGCTC3' and reverse 5'TCAGCAGGCCGAGCAGCGG3'. For connexin 43, forward 5'ATGGGTGACTGGAGCGCCTTAGGC3' and reverse 5'CTAGATCTCCAGGTCATCAGGCCG3'). The PCR profile for connexin 45 involved pretreatment at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 90 s. The PCR profile for connexin 26, 32, and 43 (35 times) was as follows: pretreatment at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 120 s. Reaction products were analyzed by electrophoresis in 1.5% (w/v) agarose gel, followed by staining of

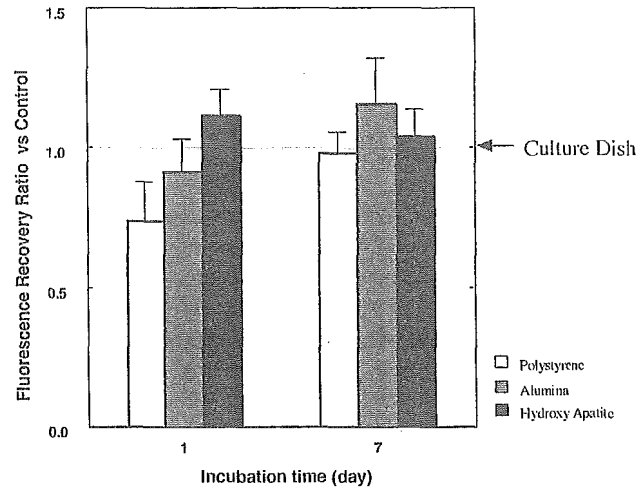


Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

the products by SYBR[®] Green I (Takara Shuzo Co., Ltd.) and detection of a 566-bp (connexin 45), 671-bp (connexin 26), 852-bp (connexin 32), and 1149-bp (connexin 43) band, respectively. For the standardization of connexin cDNA, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample was performed using GAPDH-specific primers (forward 5'CCCATCACCATCTTCCAGGAGCGAGA3' and reverse 5'TAAGTAGGACAA-CAAGGAGGTCGTGACGACGC3'; product size 578-bp). All reactions included negative controls without cDNA.

Statistical analysis

All data were expressed as the mean value ± the standard error of the means of the obtained data and treated statistically with Student's *t* test.

RESULTS

Figure 1 shows effects of various microspheres on GJIC of NHOst in contact with the microspheres for 1 and 7 days. The microspheres were precoated on 35-mm culture dishes before cell seeding. When the NHOst were cultured with precoated PS, PE, and alumina microspheres, their GJIC level was similar to that in NHOst cultured on a normal culture dish. On the other hand, the GJIC level was 1.5 times that of NHOst on the normal dish when they were cultured with precoated hydroxy apatite microspheres. After 7 days, the GJIC of NHOst in contact with microspheres became similar to that of normal NHOst, irrespective of the type of microsphere. The change in GJIC of NHOst in contact with added microspheres is shown in Figure 2. As seen in Figure 1, hy-

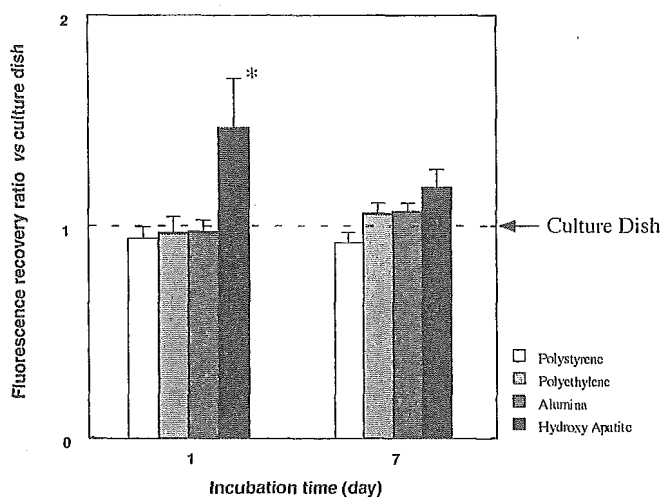


Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (**p* < 0.01 against culture dish).

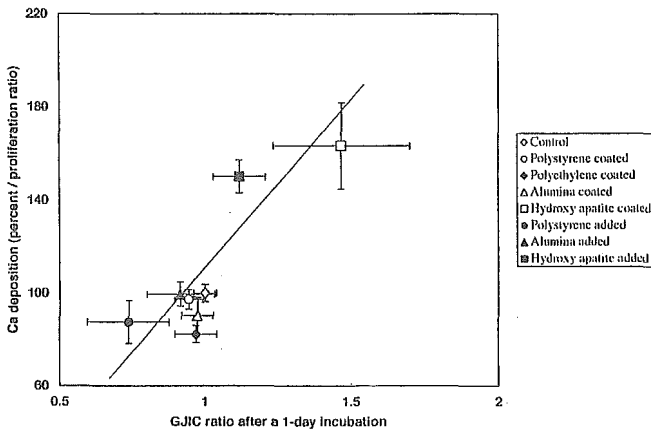


Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres ($r^2 = 0.74$).

droxy apatite microspheres enhanced their GJIC after a 1-day culture compared to cells on a normal plate. The degree of enhancement of GJIC is, however, smaller than that seen in NHOst in Figure 1, and no significant difference was observed between NHOst in contact with the hydroxy apatite microspheres and those cultured without microspheres. In addition, Figure 2 indicates that addition of PS microspheres into a culture of NHOst inhibited GJIC.

To consider the effects of tested microspheres on not only GJIC but also the differentiation of NHOst, changes in the amount of calcium deposited after a 1-week coculture of NHOst with various microspheres were estimated. From Figure 3, it is suggested that there is the possible relation between the GJIC of NHOst cocultured with microspheres for 1 day and

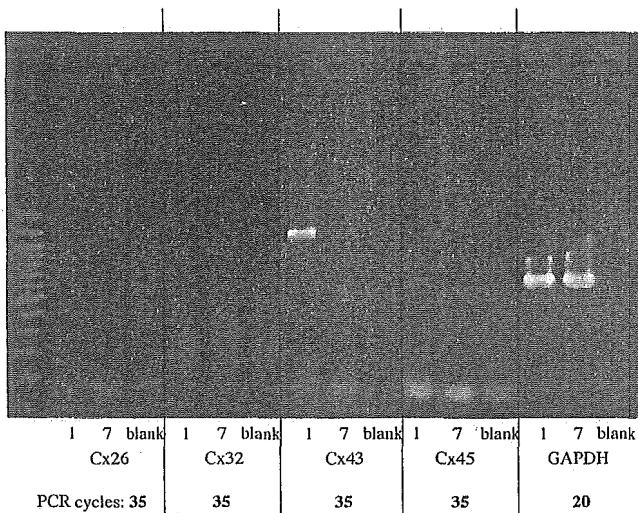


Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2×10^5 . RT-PCR cycles of each lane are expressed at the bottom of the figure.

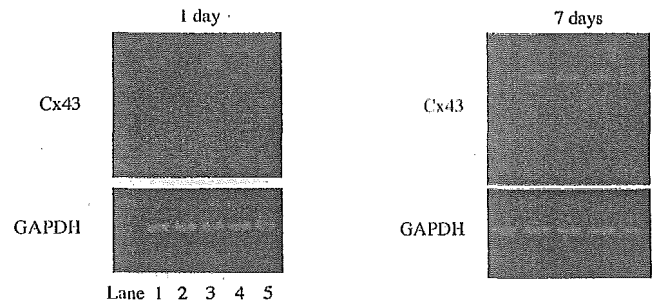


Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

the amount of calcium deposited after a 1-week coculture with the same microspheres.

To clarify which connexins exist in NHOst, we performed RT-PCR to detect mRNA of connexin 26, 32, 43, and 45 in NHOst cultured on a normal culture dish. Figure 4 shows the result of RT-PCR to amplify the mRNA from whole RNA collected from NHOst cultured for 1 and 7 days. As shown in the figure, only connexin 43 and 45 were detected in NHOst. When cells were cultured for 7 days, connexin 43 was detected at a lower level than that detected after the 1-day culture, while connexin 45 was not detected.

Figures 5 and 6 show the results of RT-PCR to amplify mRNA of connexin 43 and 45 in NHOst cultured with various precoated microspheres. The NHOst cultured with microspheres did not express mRNA of connexin 43, except those with PE microspheres. After 7 days, the expression was suppressed in the normal NHOst while the expression was observed in NHOst cultured with microspheres, irrespective of kind of the microsphere. On the other hand, mRNA expression of connexin 45 was suppressed after a 1-day culture of NHOst only with alumina microspheres, followed by a decrease in expression of the mRNA after their 7-day culture.

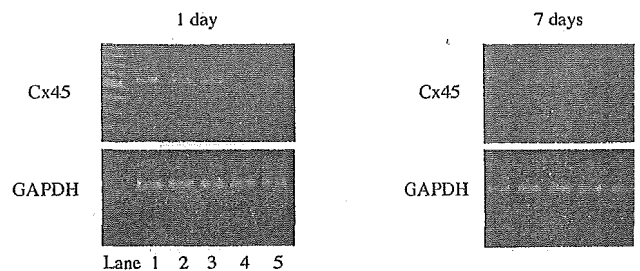


Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

DISCUSSION

As shown in Figure 3, normal human osteoblasts (NHOst) in contact with microspheres showed different levels of calcium deposition only after a 1-week culture, suggesting composition of the microspheres affects NHOst differentiation level. The differentiation was suppressed by the contact with PS, PE, and alumina microspheres, while HA microspheres showed the potential to enhance the differentiation. It has been reported that GJIC plays an important role in not only the homeostasis of cells but also their differentiation.¹⁹⁻²² In addition, GJIC is affected by the microsphere's composition, as has been reported using a fibroblast cell line.¹⁸ Therefore, the results shown in Figures 1 and 2 suggest that the enhanced differentiation of NHOst relates to GJIC enhancement on a 1-day culture in contact with HA microspheres, especially the precoated microspheres. In addition, on coculture with other microspheres, GJIC was slightly suppressed at 1 day, although no significant difference compared to control NHOst was observed. We have already studied effects of the microspheres on NHOst differentiation, and enhancement of calcium deposition by coculture with the hydroxy apatite microspheres was observed. Figure 3 suggests a relationship between the calcium deposition and GJIC on day 1. This also indicates that GJIC of the NHOst, in contact with materials in the microsphere form, in the early stage may be one factor affecting their differentiation.

It has been reported that GJIC of cells derived from human osteoblasts is mainly composed of connexin 43 and 45.^{22,26,27} In this study, it is also indicated that GJIC of NHOst is composed of connexin 43 and 45 (Fig. 4). Therefore, it is possible that changes in the level of their GJIC is ascribed to the change in mRNA expression level of connexin 43 and 45 and their expression ratio. From Figures 5 and 6, mRNA of connexin 43 was expressed only in normal NHOst and those cultured with PE microspheres, while it was slightly expressed in NHOst cocultured with HA. On the other hand, mRNA of connexin 45 was expressed in NHOst in all conditions, except those cocultured with alumina microspheres. Because HA was observed to enhance GJIC of NHOst, this suggests that connexin 45 may play a role in GJIC at an early stage. This also suggests that a higher level of connexin 45 than that of connexin 43 may be important in the enhancement of GJIC. However, although the mRNA expression of neither connexin 43 nor 45 was observed in NHOst cocultured with alumina microspheres, their GJIC was similar to that of normal NHOst. Moreover, it has reported that gap junctions formed by connexin 43 are more permeable to negatively charged dyes such as lucifer yellow, calcein, and carboxyfluorescein used in this study, more than those formed by

connexin 45, and an increase of connexin 43 expression and GJIC function parallel osteoblast differentiation.^{22,28} These are inconsistent with our findings and indicate that not high expression, but a rapid decrease of connexin 45 mRNA is probably very important for GJIC change and differentiation of the osteoblasts. Therefore, even though connexin 45 may play an important role in the early stage of GJIC in NHOst, it is probable that another connexins or other mechanisms of GJIC play a role in the GJIC of NHOst.

Because many proteins are involved in GJIC formation,²⁸ other mechanisms or proteins may be important in the GJIC change induced by the contact with the microspheres. It has reported that cadherins, which are important proteins for form tight junction between cells, control connexin 43-mediated GJIC.^{29,30} In addition, a microtubule network inside a cell has been reported to play an important role as guidance for delivery of connexons, which are composed of six connexin molecules, to the cell membrane to make gap junctions.³¹ Usually, surface characteristics of materials affect cell attachment as well as cell morphology, suggesting signal cascades of cell attachment and cytoskeleton rearrangement in the cell were influenced by the characteristics. Therefore, it is probable that a surface characteristic of the microspheres affect these molecules in NHOst, resulting in changes of GJIC activities. Further studies on changes in not only connexin molecules but also other molecules such as cadherin, actin, and microtubule in NHOst, is necessary to clarify the mechanism of GJIC. In the future, we will study the above, and find another molecules participating in the GJIC of NHOst and the mechanisms regulating the connexins in NHOst.

In conclusion, the GJIC level of NHOst changes on contact with microspheres, and is affected by the composition of the microspheres. The GJIC level in the early stage might be important in the differentiation control of NHOst and the level may be controlled partly by expression of connexin 43, connexin 45, and unclarified connexins in addition to other mechanisms regulating GJIC function. Detecting a biomaterial's effect on the GJIC of human cells may be one useful method for estimating its biocompatibility.

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

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Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using *in vitro*

techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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