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Cytotoxicity of Various Calcium Phosphate Ceramics

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Abstract. The cytotoxicity of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), was investigated. Based on the guidelines of biological test for medical devices in Japan, a cytotoxicity test of these calcium phosphates was carried out using Chinese hamster V79 lung fibroblasts. The cytotoxic study revealed that FAp and α-TCP showed high cytotoxicities. From various analyses, it was considered that the cytotoxicity of the FAp was due to fluorine ions extracted in a culture medium and the cytotoxicity of α-TCP resulted from a decrease in pH of the medium by the phosphoric acid, which produced by hydrolysis of the α-TCP.

Introduction

From the view point of biological affinity to bone, calcium phosphate (CP) ceramics have been studied to utilize for many purposes in a medical field. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP), are known to be biologically bonded to natural bones and their porous materials have been studied for effective restoration of bone defects.[1,2] Fluoroapatite ($Ca_{10}(PO_4)_6F_2$, FAp) has been reported to have a potential of novel bone repairing materials with high stability *in vivo*, since solubility of FAp is lower than that of HAp.[3,4] In addition, CP cement is also promising for bone repair and it is well known that α-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) and tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement.[5,6]

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical and chemical property, should be satisfied. Among them, biological safety is important for the biomaterials. Since only a few studies which discuss the cytotoxicity of calcium phosphate ceramics have been reported, the cytotoxicity of CP ceramics is worthy to be investigated in order to design bioceramics with good biological safety for medical application. Therefore, the cytotoxicities of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP) were investigated.

Materials and Methods

Materials

Five kinds of CP ceramics, HAp, FAp, α-TCP, β-TCP and TTCP were purchased from Wako chem. Co. Ltd. CP powders (0.25 g) was put into stainless mold and uniaxially pressed at 30MPa for 1 min to form a pellet. The dimensions of the obtained CP pellet were 1mm in thickness and 12mm in diameter. CP pellets were sterilized by an autoclave at 121°C for 20 min.

Cytotoxicty test on CP ceramics

Cytotoxicity test was carried out using Chinese hamster V79 lung fibroblasts by a colony assay system. V79 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd.) with 10% fetal calf serum (FCS, Intergen Co. Ltd.) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The method of cell seeding in the cytotoxicity test of CP ceramics was shown below; each CP pellets were placed in each culture wells of 24 well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well. Then, 50 cells/300μl of the cell suspension in the

culture medium were added into each well and incubated at 37°C for 4 h. Finally, 400μl of the culture medium was added into each well and the plates were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days. In order to investigate a cell adhesive property on the CP ceramics, the culture medium was changed after 4 h and further incubated for 7days. The removed culture medium was transferred to another well of a new plate and incubated for 7days as well.

Cytotoxicity of extracts from CP ceramics was also investigated in this study. Suspensions of CP ceramics in the culture medium (100mg/mL) were stirred at 37°C for 3days under the rotation condition at 150rpm. The suspensions were centrifuged and the supernatants were collected as test extracts. In addition, media with various pH values were prepared using HCl solution to investigate an effect of pH on cell survival. Fifty V79 cells in 1ml of the extracts or the medium with different pH value were incubated at 37 °C for 7days.

After 7-day incubation, the cells were fixed in methanol and the number of the V79 colonies was counted after staining cells with 5%-Giemsa solution to estimate the cytotoxicity of the test sample. In addition, the pH of the medium after 7-days culture was measured to estimate the effect of the pH of the medium on the cytotoxicity test.

Characterization of CP ceramics

The structural changes of CP before and after an autoclave-sterilization or an incubation at 37°C culture were investigated by powder X-ray diffraction (XRD) analysis and scanning electron microscopy (SEM). XRD analysis was carried out (Rigaku Co., Ltd. / RINT 2000) with the CuK_α radiation at 40kV, 50mA. SEM observations were performed (JEOL / JSM-5800LV) with an accelerating voltage of 25kV.

Results and Discussion

Cytotoxicity of various CP ceramics

From XRD analysis, no structural changes of CPs were observed after an autoclave sterilization. After staining CP pellets, it was observed that cell colonies were formed on various CP ceramics pellets (Fig.1(a)). The results of the cytotoxicity test of CPs are shown in Fig.1(b). The cell colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colonies formed on these pellets against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colonies on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. As no colonies were observed after 7-day culture of the removed medium in cell adhesion studies of CP ceramics, these results suggested that V79 cells can adhere and be viable on these pellets, irrespective of the type of CP ceramics. Figure 2 shows the formation of colonies cultured in extract from CP ceramics. The cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicities was similar to that of the cytotoxicities on the respective CP pellets themselves (Fig.1(b)).

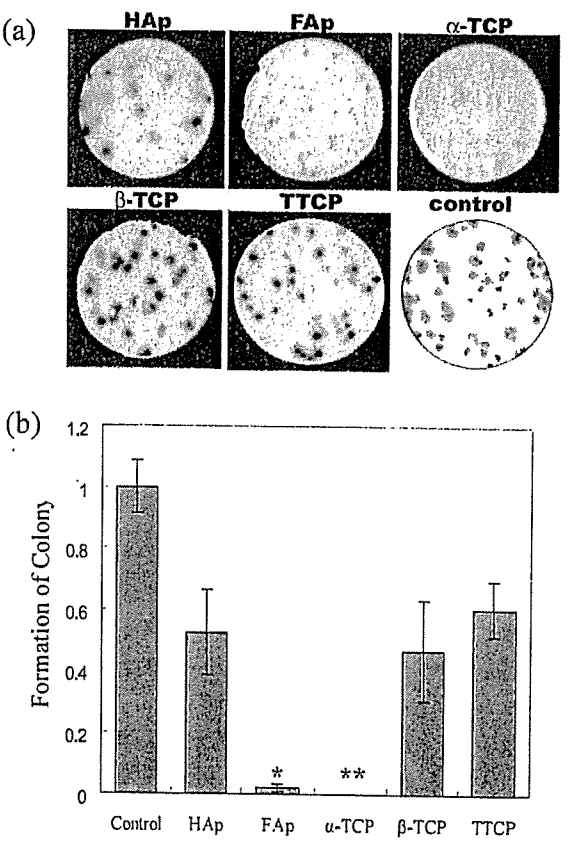

Fig.1. The appearance of colonies on various CP pellets (a) and their colony formation ratios (b). (*$p<0.05$ against for V79 alone, **$p<0.01$ against for V79 alone)

The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are highly cytotoxic. In addition to results shown in Fig.2, it is suggested that the differences in the colony formation ratio on various CP pellets are ascribed to difference in extract properties from the CP, which may be related with the composition or crystal structure. As shown in Table 1, the pH of culture medium after incubation with FAp pellets is almost the same as that of HAp, while the pH of the α-TCP-incubated medium is much lower than that of the other CP ceramics-incubated media. In order to consider the reason of the low pH of the a-TCP-incubated medium, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP before and after extraction treatment are shown in Fig.3. Before extraction, a particle size of α-TCP was about 10μm and its surface was smooth (Fig.3(a) and (b)). However, whisker-like precipitates of 1-2μm in length and 2-300nm in width were observed at the surface of α-TCP after the extraction, although there was no change in its particle size (Fig.3(c) and (d)). It is well known

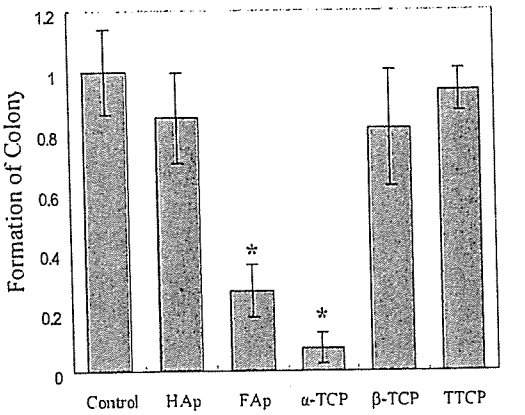

Fig.2. Formation of colony cultured in extract from various CP ceramics. (*p<0.01 against for V79 alone)

Table 1. The pH and Ca concentration of culture medium after incubation.

Samples	pH of medium after culturing	Ca concentration /ppm
V79 alone	7.12	-
HAp	7.24	0.19
FAp	7.20	0.17
α-TCP	6.76	72.62
β-TCP	7.40	1.27
TTCP	7.65	0.58

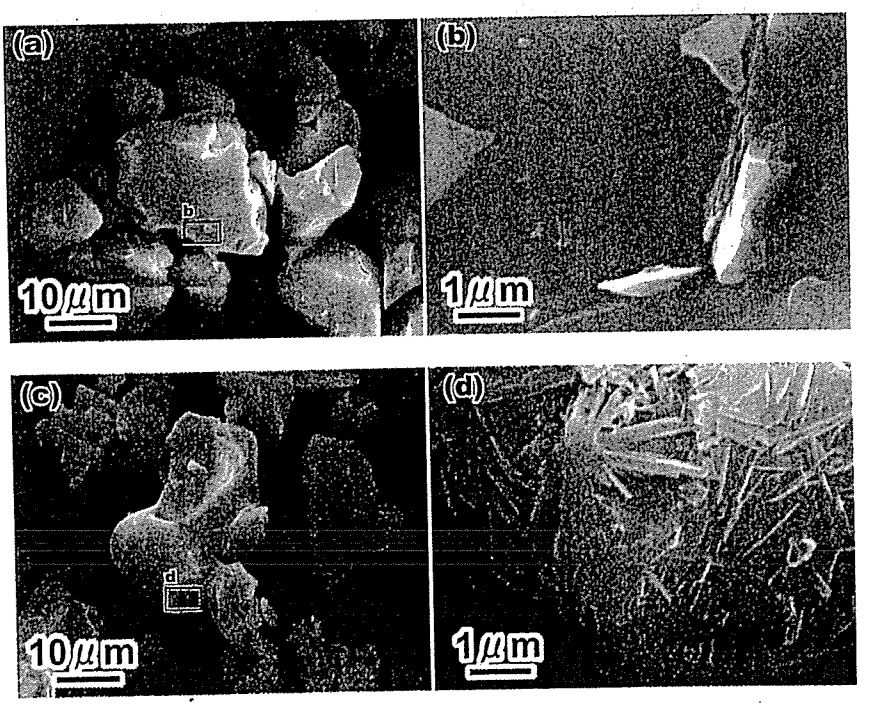

Fig.3. SEM images of α-TCP before (a), (b) and after extract treatment (c), (d). (a) and (c) are whole image of before and after extract treatment, respectively. (b) and (d) are enlarged image of the area enclosed by a rectangle in (a) and (c), respectively.

that calcium phosphates convert to HAp in aqueous solution with high pH value. Since the solubility of α-TCP is higher than that of other calcium phosphates, α-TCP rapidly converts to HAp as follows;

$$10Ca_3(PO_4)_2+6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2+2H_3PO_4$$

According to the report of this conversion [7], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates in Fig.3 (d) can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation.

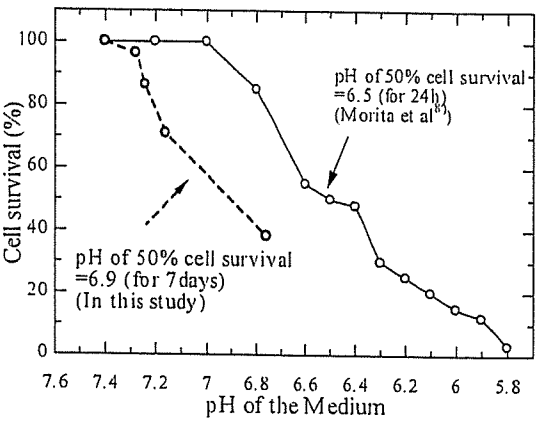

Fig.4. V79 cell survival in the medium with various pH values.

In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid causes the decrease in pH of the solution. As shown in Fig.4, Morita and co-workers[8] have reported that low pH itself could be clastogenic to mammalian cells and the pH of 50% V79 cell survival was 6.5 for 24h incubation. In the present colony assay system, the pH of 50% V79 cell survival was 6.9 for 7-days incubation. In addition, we confirmed that phosphoric acid showed no or weak cytotoxicities under our present experimental conditions. Therefore, it is suggested that the cytotoxicity of α-TCP is mainly due to the pH decrease resulting from an increase of the phosphoric acid ion by the hydrolysis conversion from α-TCP to HAp.

On the other hand, FAp has the same crystal structure of HAp but the hydroxyl ions in HAp substituted by fluorine ions. Since it is probable that difference of the colony formation on various CP ceramics are due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp. In conclusion, this study has revealed that FAp and α-TCP have a cytotoxicity, while TTCP has lower cytotoxicity than other calcium phosphates. To develop biomaterials made from calcium phosphate, further studies are necessary to clarify their cytotoxic mechanisms.

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

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

2. Materials and methods

2.1. Materials

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

2.2. Preparation of media and culture dishes

The HA polysaccharide was dissolved in distilled water at a concentration of 4 mg/ml. Each of the 35-mm culture dish (Falcon 1008, Becton Dickinson) was coated at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. The HA-coated dishes were dried further under sterile air flow at room temperature for 12 h before use. In order to investigate the effect of HA-addition on the functions of HIT-T15 cells, different media were prepared at a final concentration of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml. HA-treatment is performed to cells for 24 h.

2.3. Cells and cell culture

A hamster pancreatic β-cell line, HIT-T15 (HIT-T15 cells, Dainippon Pharmaceutical Co., Japan), was cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 IU penicillin-G and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The subculture cells were seeded at a density of $1.0–5.0 \times 10^5$ cells/ml in multiwell plates or culture dishes. When they reached more than 80% confluence, the cells were used for various studies. Throughout the cell growth period the culture media were replaced every 2 days.

2.4. Measurement of cell viability

To evaluate the affect of HMW HA on cell viability of HIT-T15 cells, HIT-T15 cells (1×10^5) were incubated into the various concentrations of HA-coated 24-well plates, or after the cells were seeded onto 24-well plates and pre-incubated in a 10% FBS/RPMI 1640 medium overnight, the medium was exchanged for 10% FBS/HA/RPMI 1640 medium prepared. After 24 h of HA-treatment, the cell viability was determined by the WST-8 reduction assay, according to the manufacturer's instructions. Control cells received fresh medium without HA.

2.5. Measurement of insulin release and insulin content

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

2.6. Measurement of dye transfer

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

2.7. Western blot analysis

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

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

3. Results

3.1. Cell viability

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

3.2. Insulin secretion and insulin content

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

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

3.3. Dye transfer

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

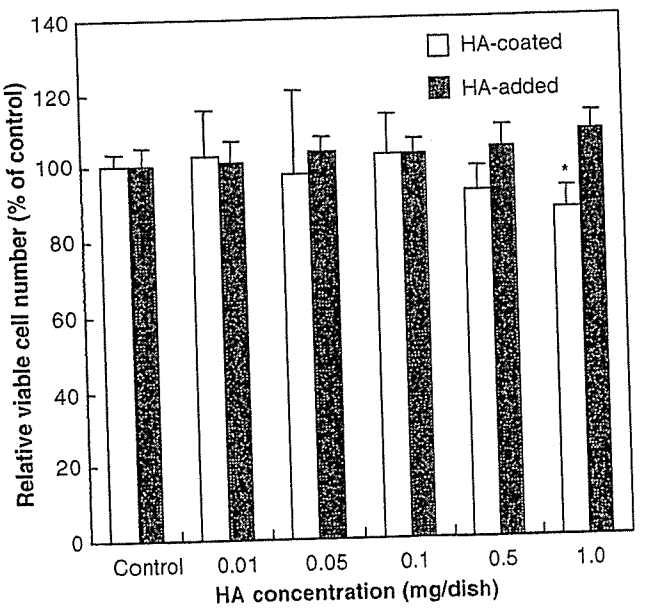


Fig. 1. Concentration-dependent effects of HA-treatment on viability of HIT-T15 cell. After HIT-T15 cells were incubated with HA-coated or HA-added for 24 h, the viable cell numbers of HIT-T15 cell were determined by WST-8 assay as described in methods. Each value denotes the mean±S.D. of three separate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

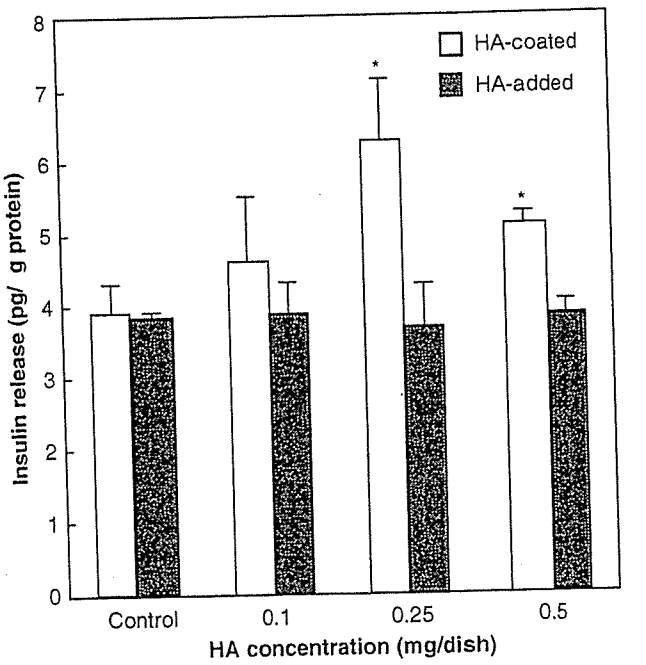


Fig. 2. Insulin secretion from HIT-T15 cells by HA-treatment. HIT-T15 cells were incubated with HA-coating (□) or HA-added (■) for 24 h and then stimulated for 60 min with 11.1 mM glucose in KRB buffer. The released insulin in the spent medium was determined by ELISA insulin kit. Each value denotes the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

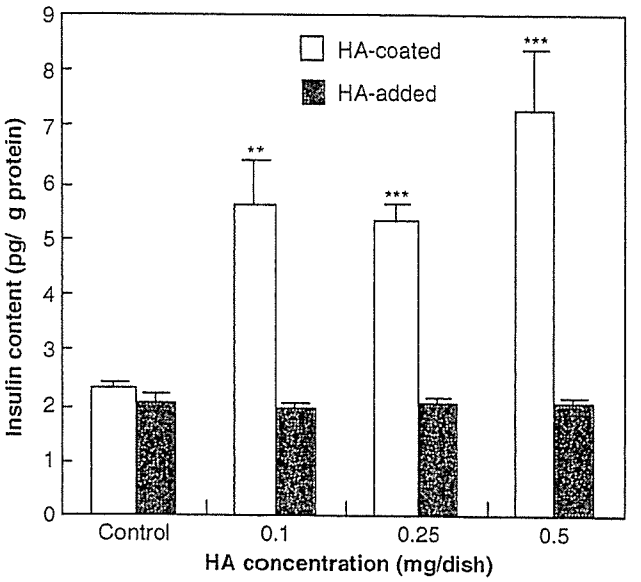


Fig. 3. Insulin content of HIT-T15 cells incubated with HA-coated (□) and HA-added (■). Cells were incubated in the presence of different HA concentrations (0.1–0.5 mg/dish) for 24 h and then stimulated for 60 min with 11.1 mM glucose. The insulin content in the extracts was determined by ELISA insulin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

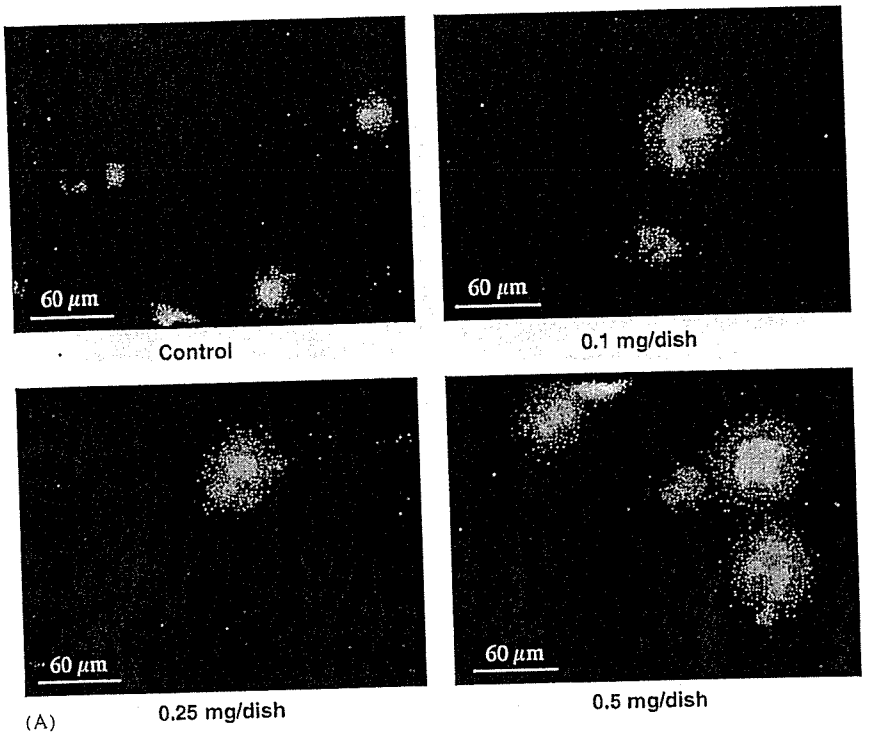
3.4. *Cx43 expression*

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



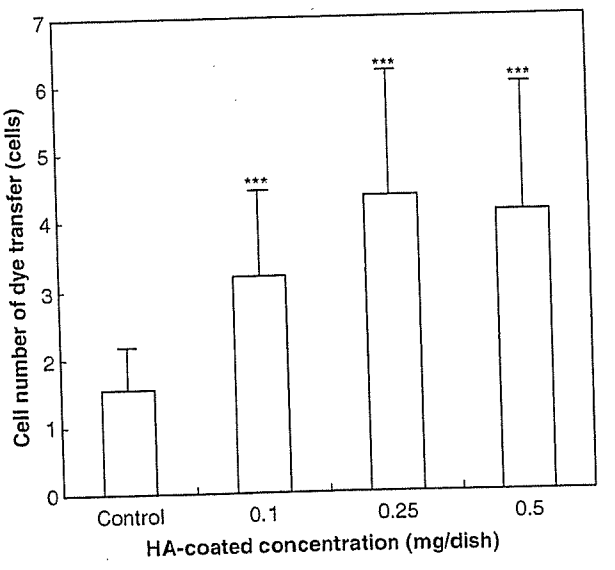


Fig. 4. Concentration-dependent effects of HA-coating on dye transfer in HIT-T15 cells. Cell adherent to glass coverslips were microinjected with 4% Lucifer yellow. Transfer of dye to neighboring cells was assessed by epifluorescence microscopy 2 min later. This is a representative expression of 18 injections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

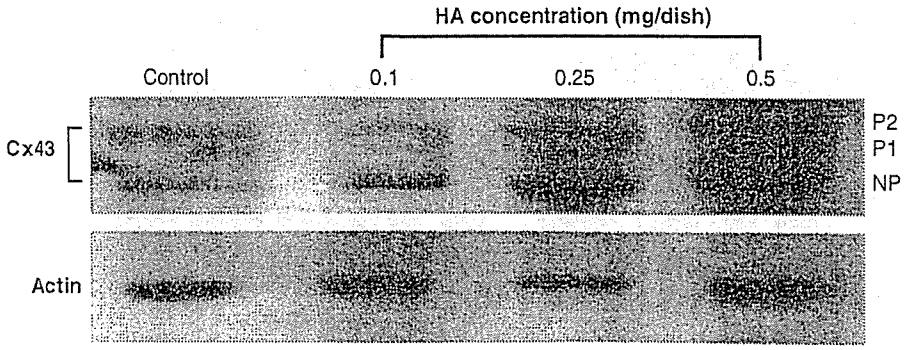


Fig. 5. Identification of Cx43 in HIT-T15 cells grown on the HA-coating dish by Western blot analysis. After HIT-T15 cells were incubated into HA-coated dish for 24 h, cells were lysed and proteins (20 μg) were separated by SDS–PAGE followed by Western blotting using rabbit anti-Cx43 antibody. Actin 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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STUDIES ON THE EFFICACY, SAFETY AND QUALITY OF THE TISSUE ENGINEERED PRODUCTS: ENHANCEMENT OF PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS BY THE NEW POLYSACCHARIDES

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Abstract: Human mesenchymal stem cells (hMSCs) have the capacity to proliferate and differentiate into multiple cells etc. Polysaccharides can modulate the cell proliferation of human endothelial cell. Here, we investigated the role of different kinds of new polysaccharides to regulate the gap junctional intercellular communication (GJIC) and cell proliferation of cultured normal human dermal fibroblasts (NHDF) cells and hMSCs. The NHDF cells and hMSCs were cultured for 4 days with new polysaccharides. The cultures were then analyzed to verify the extent of GJIC by the scrape-loading dye transfer (SLDT) method, using Lucifer yellow. Alamar blue staining was performed to determine the proliferation of the cultured cells. In NHDF cells, the GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides. On the contrary, in hMSCs, the GJIC was slightly inhibited in all cultured treated cells. But proliferation was enhanced in both cells with different polysaccharides, the extents of cell proliferation was stronger in hMSCs than in NHDF cells. These findings reveal that new polysaccharides seem to play an important role in hMSCs, thus provide a novel tool on tissue engineering.

Key words: GJIC, Proliferation, NHDF, hMSCs.

1. INTRODUCTION

Human mesenchymal stem cells (hMSCs) are multipotent cells have the capacity to proliferate and differentiate into bone, cartilage and adipocytes, and are useful for human cell and gene therapies [1]. Polysaccharides are macromolecules formed from many sugar units connected by glycosidic

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

linkages. It has two basic functions: serve for monosaccharide storage to make cellular energy and serve as structural components. Sulfated polysaccharide was reported to cause modulation of human endothelial cell proliferation [2]. Sweeney *et al.* also reported that sulfated polysaccharide increases and mobilizes hematopoetic stem cells in mice and nonhuman primates [3]. Furthermore, the inhibition of GJIC can disrupt the balance of cell homeostasis, leading to increase cell proliferation [4]. The aim of this study is to investigate the ability of different kinds of new polysaccharides to regulate the GJIC and cell growth of cultured NHDF cells and hMSCs.

2. MATERIALS AND METHODS

2.1. ***Materials:*** 4 different kinds of polysaccharides were used in this experiment.

2.2. ***Cell Culture:*** The NHDF cells were obtained from Asahi Techno Glass (Tokyo, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5 % CO_2 atmosphere at 37°C. The hMSCs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, USA), and maintained in mesenchymal cell growth medium (MSCGM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C.

2.3. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Cells $1x10^5$/ml (2ml medium/dish) were seeded on to the 35 mm dishes. After 4 hr seeding in a 5% CO_2 atmosphere at 37°C, different kinds of new polysaccharides at the concentration of 2mg/ml, 1ml per dish (35mm dish) was added and incubated at 37°C for 4 days. Then, confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS (+) and the extent of dye migration length was measured using fluorescence microscope.

2.4. ***Proliferation assay:*** $4x10^4$ (0.5 ml medium/well) cells per well of 24 well culture plate were seeded. After 4 hr seeding in a 5% CO_2 atmosphere at 37°C, different kinds of new polysaccharides at the concentration of 2mg/ml was added and incubated at 37°C for 4 days. Then, cell proliferation was quantitatively measured by alamar blue (Biosource International, Inc., Camarillo, CA) assay. The assay showed the metabolic activity of the cells by detection of mitochondrial activity. Here, alamar blue used as the indicator dye, was incorporated into the cells, reduced and excreted as a fluorescent product. At the end of 4 days culture, the media from all wells were discarded, and filled with 1 ml/well of 1:20 of alamar blue/fresh medium. The culture plates were incubated at 37°C for 4 h. After the incubation period, two aliquots of 100 µl of solution from each well were transferred into new wells of a Costar 96-well

microplate of tissue culture (Costar type 3595, Corning Co. Ltd.). Equal volume of fresh medium per well (total four wells) served as blanks. The extent of cell proliferation was quantitated by Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535-nm excitation and 590-nm emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from the experimental values to eliminate background readings.

2.5. *Statistical analysis:* Student's *t* test was used to compare the implanted samples with the controls. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean±SD.

3. RESULTS

NHDF cells: In NHDF cells, GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides (**p < 0.01) (Figure 1A). But the cell proliferation was significantly increased in cells treated with different kinds of polysaccharides (**p < 0.01) (Figure 1B).

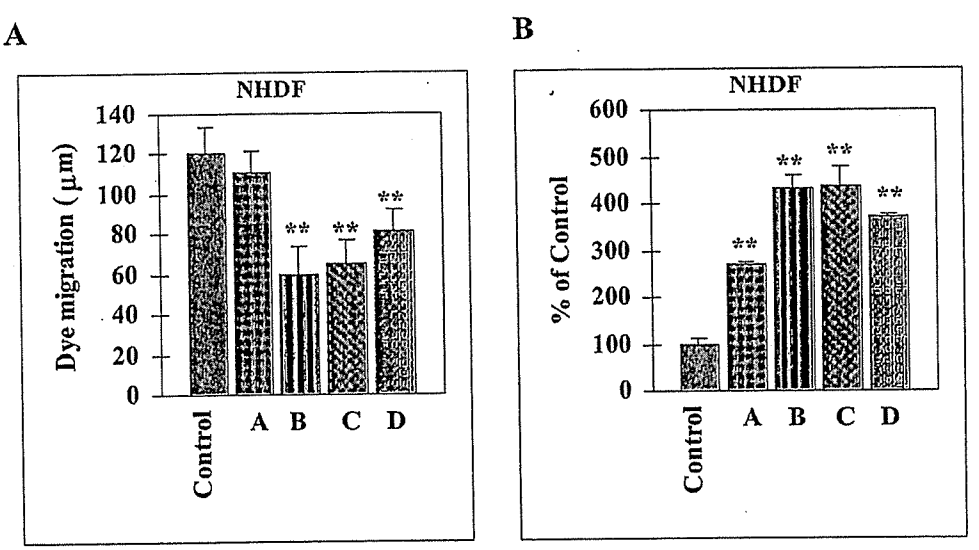

Figure 1. In A, Statistical analysis of SLDT assay and in B, cell proliferation of NHDF cells. ** p < 0.01.

hMSCs: In hMSCs, GJIC was also inhibited in all treated cells but significantly in only treated with "D" (*p < 0.05, **p < 0.01) (Figure 2A). Here proliferation also was significantly enhanced in cells treated with different kinds of polysaccharides (**p < 0.01) (Figure 2B). But stimulatory reaction was much more in hMSC cell than NHDF cell.

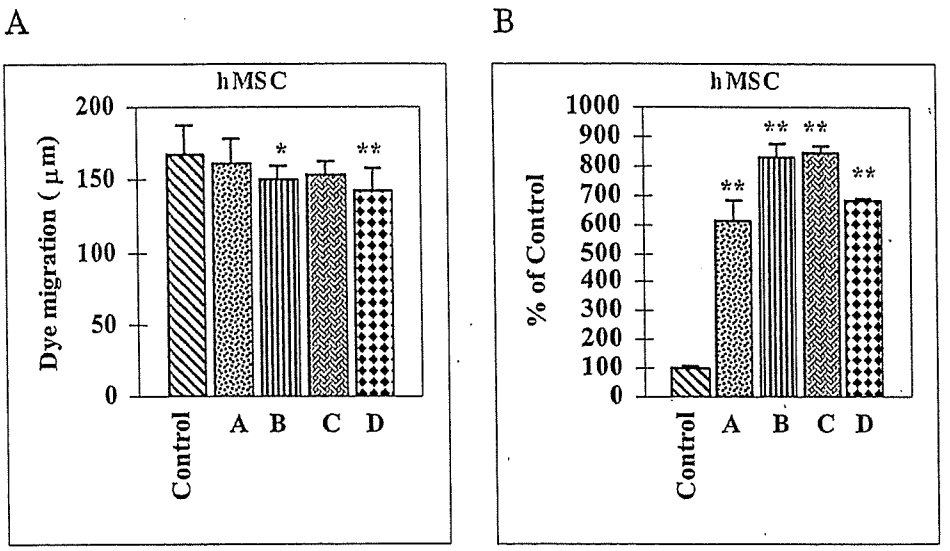

Figure 2. In A, Statistical analysis of SLDT assay and in B, cell proliferation of hMSCs. * $p < 0.05$, ** $p < 0.01$.

4. DISCUSSION

hMSCs are used for tissue engineering of bone and cartilage and provide a versatile model system to study mesenchymal proliferation. In this study we identify several distinct roles of new polysaccharides in hMSC biology, which disclose a role of polysaccharides in hMSC proliferation. GJIC was significantly inhibited in cells treated with different kinds of new polysaccharides in NHDF cells. But in hMSCs, GJIC was slightly inhibited in all cultured treated cells. In contrast, cell proliferation was enhanced by different polysaccharides in hMSCs (6 to 8 folds) more than in NHDF cells (2 to 5 folds) in comparison with controls. As stated earlier, in mice and monkeys, sulfated polysaccharide such as fucoidan caused increase in hematopoetic stem cells [3] and Matsubara *et al.* reported that basement membrane-like extracellular matrix (bmECM) had greater effects on the proliferation of hMSC [5]. Our result also coincided with these reports. Usually, inhibition of the function of connexin is considered to cause the cellular proliferation [4]. Therefore, these findings, that there is a relationship between the inhibitory effects on the connexin function and cellular proliferation, coincided with the result previously reported. Our studies postulated that these new polysaccharides seem to play a significant role in cell proliferation of both NHDF cells and hMSCs. Especially, these new polysaccharides are novel materials to increase the cell number of hMSCs and therefore hMSCs provide a good and clinically relevant model system. In addition, the positive effect of new polysaccharides on hMSC proliferation warrants further studies toward its exploitation in tissue engineering.

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STUDIES ON THE EFFICACY, SAFETY AND QUALITY OF THE TISSUE ENGINEERED PRODUCTS: EFFECTS OF A CATALYST USED IN THE SYNTHESIS OF BIODEGRADABLE POLYMER ON THE CHONDROGENESIS OF HUMAN ARTICULAR CARTILAGE

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Abstract: Among different synthetic biodegradable polymers, polyesters such as poly (glycolic acid) (PGA) is an attractive candidate in orthopedic applications, because of its degradation product glycolic acid is a natural metabolite. The biocompatibility of PGA that was synthesized with and without inorganic tin catalyst, in chondrogenesis of human articular cartilage (HAC) was investigated using a 4 weeks micromass culture system. PGA with tin catalyst caused significant enhancement in chondrocyte proliferation and expression of collagen type II gene. Amounts of total collagen and collagen type II protein were also increased. However, aggrecan gene expression was almost similar to control cultures. On the contrary, PGA without catalyst caused an inhibitory action on the chondrogenesis. From the viewpoint of safetyness, PGA was not suitable to use as the biodegradable scaffold for cartilage.

Key words: Human articular cartilage, Chondrogenesis, PGA, Tin catalyst.

1. INTRODUCTION

The fields of biotechnology and tissue engineering by using different synthetic biodegradable polymers are general concepts because of its disappearance in the body. In general, synthetic biodegradable polymers offer greater advantage over natural or other materials. The prime advantages include the capacity to change the mechanical properties and degradation kinetics to suit various applications. Synthetic biodegradable polymers, especially polyester

S. Iijima and K.-I. Nishijima (eds.), Animal Cell Technology: Basic & Applied Aspects, 87–92.

such as poly (glycolic acid) (PGA) plays an important role in orthopedics. PGA, a polymer of glycolic acid can be synthesized under the influence of different catalysts. The common catalysts used include organotin, antimony, lead, and zinc. Organotin compounds are known agents to cause neurotoxicity [1], cytotoxicity [2], immunotoxicity and genotoxicity [3] in human and other experimental animals. Disproportionate dwarfing syndrome, affecting the limbs severely than the trunk, was observed in the rats that had been injected with certain tin compounds [4]. No study yet has reported the chondrogenic effects of PGA, synthesized with and without inorganic tin catalyst. In this study, the biocompatibility of PGA synthesized with and without tin catalyst was investigated using human articular cartilage (HAC) in a micromass culture system.

2. MATERIALS AND METHODS

2.1. ***Medium and Polymers Used for Cell Culture:*** Chondrocyte growth medium were commercially obtained from BioWhittaker, Inc., (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (Mw = 1,500) and PGA without catalyst (PGA) (Mw = 1,100) were tailor-made and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical Co. Irvine, UK).

2.2. ***Cells and Culture Methods:*** HAC of the knee joint was commercially obtained from BioWhittaker, Inc., (Walkersville, MD, USA). High-density micromass cultures were started by spotting $4x10^5$ cells in 20µl of medium onto Costar 24-well microplates for tissue culture (Costar type 3526, Corning Co. Ltd.). After 2 h of attachment period at 37°C in a CO_2 incubator, culture medium (1ml/well) was added into each well. Media were supplemented with DMSO (0.8 µl/ml), PGA and PGA (Sn) (50 µg/ml). HAC cultured with DMSO was used as control. The cultures were continued for 4 weeks with medium change twice in a week. At least four cultures were run for each sample.

2.3. ***Cell Proliferation Study:*** Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Ltd., Osaka, Japan) staining method. After 4 weeks culture, cells were fixed with 100% Methanol, stained by applying 0.1% crystal violet in Methanol, and washed. Again methanol was applied and the absorbance was measured at a wavelength of 590 nm using an ELISA reader (Bio-Tek Instruments, Inc.,Winooski, Vt., USA).

2.4. ***Differentiation Assay*:** After proper washing with methanol and acetic acid, proliferation assay was followed by the differentiation assay by

staining the cells with 1% (v/v) alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4-M guanidine hydrochloride (GH) and the bound dye was measured at wavelength of 600 nm using an ELISA reader (Bio-Tek Instruments, Inc.,Winooski, Vt., USA).

2.5. ***Analytical Assays:*** Commercially available assay kit [collagen assay kit, Biocolor Ltd, Newtownabbey, Northern Ireland] was used for the measurement of collagen within the cultured cells as previously described [5]. The amounts of total collagen content (acid and pepsin soluble fractions) and collagen type II protein of the cultured chondrocytes was detected as per manufacturer's instruction. The absorbance of the samples was measured at a wavelength of 540 nm using a spectrophotometer.

2.6. ***Real-time polymerase chain reaction (PCR):*** For detection of the presence of proteoglycans, namely collagen type II and aggrecan, single stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently real-time PCR was done using LightCycler system with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Penzberg, Germany). LightCyclerTM- Primer set (Roche Diagnostics) was used for quantitative detection of Collagen type II gene, aggrecan gene, and also a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

2.7 ***Statistical Study:*** Student's t test was used to compare the sample results. Statistical significance was accepted at $p < 0.05$. All values in this study are reported as means ± S.D (standard deviation).

3. RESULTS

3.1. ***Cell Proliferation and Differentiation:*** Cell proliferation was 1.8 ($^*p < 0.05$)-fold increased in PGA (Sn) treated culture compared with DMSO group as the control. Whereas cell proliferation in PGA treated culture was almost similar to DMSO group (Figure 1A). In the case of cell differentiation, PGA (Sn) group showed a slight decrease in cell differentiation compared to DMSO control (Figure 1B).

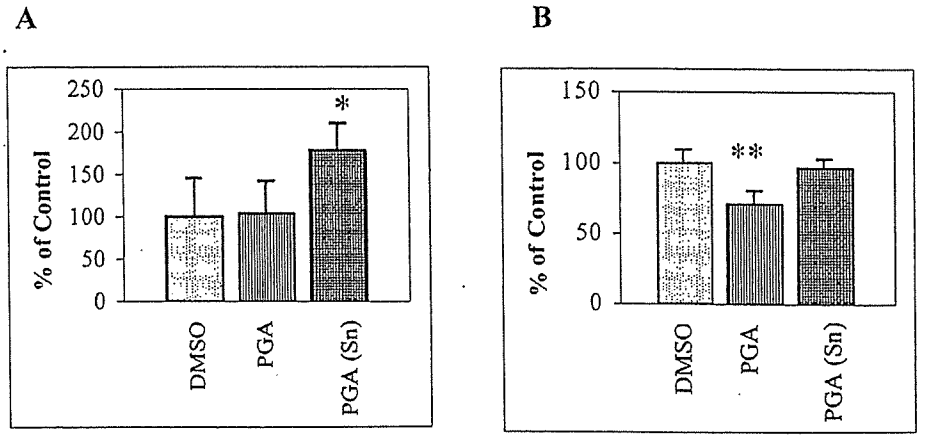

Figure 1. Cell proliferation (A) and cell differentiation (B) of human articular chondrocytes after 4 weeks culture period. * $p < 0.05$, ** $p < 0.01$.

3.2. ***Extracellular matrix gene expression:*** Collagen type II gene was strongly expressed in PGA (Sn) than in PGA and control group (Figure 2A). However, aggrecan gene expression was inhibited in the PGA and no difference was observed between PGA (Sn) and the control group (Figure 2B).

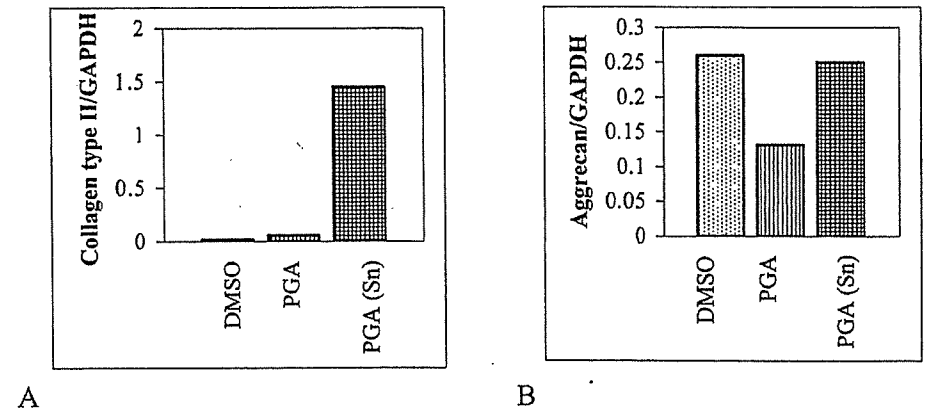

Figure 2. Expression of collagen type II gene (A) and aggrecan gene (B) in cultured chondrocytes, estimated by real time PCR method.

3.3. ***Measurement of Collagen type II protein and Total collagen amount:*** The amount of pepsin soluble and cartilage specific protein, collagen type II was significantly increased (**p <0.01) in PGA (Sn) group, but almost no difference in the amount was observed between the PGA and control group (Figure 3A). The amount of total collagen (both acid and pepsin soluble protein) was significantly increased (**p <0.01) in PGA (Sn) group compared with the controls. (Figure 3B).