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

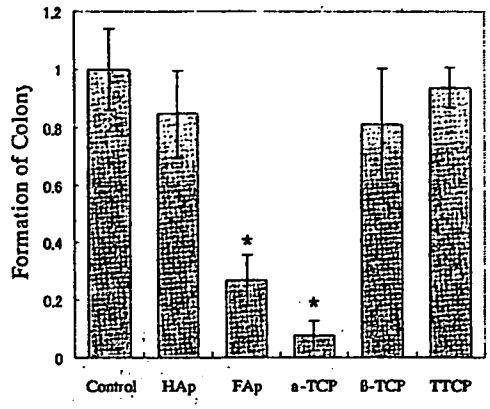

Fig.2. Formation of colony cultured in extract from various CP ceramics. (*p<0.01 against for V79 alone)

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

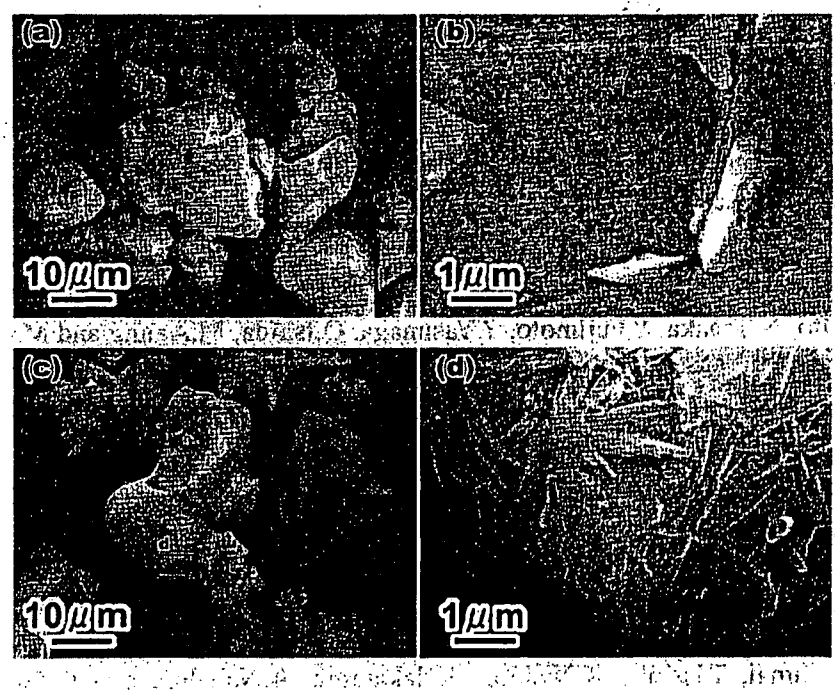

Fig.3. SEM images of α-TCP before (a), (b) and after extract treatment (c), (d). (a) and (c) are whole image of before and after extract treatment, respectively. (b) and (d) are enlarged image of the area enclosed by a rectangle in (a) and (c), respectively.

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.

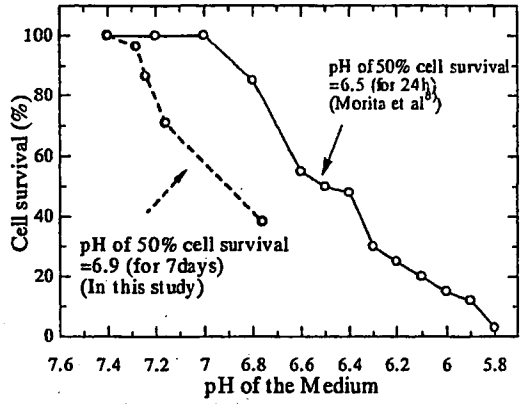

Fig.4. V79 cell survival in the medium with various pH values.

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.

Acknowledgment

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References

[1]Y.Ito, N.Tanaka, Y.Fujimoto, Y.Yasunaga, O.Ishida, M.Agung and M.Ochi: J. Biomed. Mater. Res. Vol.69A (2004), p.454
[2]Y.Wang, T.Uemiura, J.Dong, J.Tanaka and T.Tateishi: Tissue Eng. Vol.9 (2003), p.1205
[3]S.M.Barinov, F.Rustichelli, P.V.Orlovskii, A.Lodini, S.Oscarsson, A.S.Firstov, V.S.Tumanov, P.Millet and A.Rosengren: J.Mater.Sci:Mater in Med. Vol.15 (2004), p.291
[4]K.Cheng, W.Weng, H.Qu, P.Du, G.Shen, G.Han, J.Yang and M.J.Ferreira: J. Biomed. Mater. Res. B Vol 69 (2004), p.33
[5]E.L.Carey, H.H.Xu, G.C.Simon, S.Takagi and C.L.Chow: Biomaterials Vol 26 (2005), P.5002
[6]M.E.Ooms, J.G.C.Wolke, J.P.C.M.Waerden and J.A.Jansen: J. Biomed. Mater. Res.Vol.61 (2002), p.9
[7]M.Tamai, T.Isshiki, K.Nishio, M.Nakamura, A.Nakahira and H.Endoh: J. Mater. Res. Vol.18 (2003), p.2633
[8]T.Morita, T.Nagaki, I.Fukuda and K.Okumura: Muta. Res. Vol.268 (1992), p.297

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Enhancement of Differentiation and Homeostasis of Human Osteoblasts by Interaction with Hydroxyapatite in Microsphere Form

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Key words: osteoblasts, differentiation, homeostasis, hydroxyapatite, biocompatibility

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 derived from the joint. To assess the effect of wear debris on osteoblasts, we cultured normal human osteoblasts (NHOst) in contact with several kinds of microspheres as models of wear debris. The NHOst in contact with polystyrene, polyethylene, and alumina microspheres showed a lower differentiation level than NHOst alone as estimated from the amounts of deposited calcium. On the other hand, hydroxyapatite particles enhanced the differentiation of NHOst. In addition, sintered hydroxyapatite enhanced expression of osteocalcin mRNA and gap junctional communication of NHOst. This study suggests that polystyrene, polyethylene, and alumina microspheres have the potential to disorder not only the differentiation but also the homeostasis of NHOst in contact with them. However, hydroxyapatite enhanced the differentiation as well as the homeostasis of NHOst, even in microsphere form, suggesting its good biocompatibility as biomaterials for bone tissues.

Introduction

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, resulting in undesirable side effects. One good example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. Many researchers have 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 [1-3], but few researches have focused on the interaction between wear debris and osteoblasts, especially normal human osteoblasts [4]. In this study, normal human osteoblasts were cultured in contact with various kinds of microspheres made from polymers or ceramics used as model wear debris, and the effects of the microspheres' characteristics and interaction conditions were discussed in regard to the proliferation, differentiation and homeostasis maintenance of the osteoblasts.

Materials and Methods

Microspheres. Monodispersed polystyrene (PS) microspheres with different diameters (0.1, 0.5, 1, 5, and 10 μm) were kindly supplied by Japan Synthetic Rubber Co., Ltd. (Tokyo, JAPAN). Low-density polyethylene (PE) microspheres were kindly provided 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 and un-sintered hydroxyapatite (HAp) microspheres (7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. (Chiba, Japan). Determined by Multisizer II (Coulter Electronics Inc., Hialeah, FL), the average diameters of PE and alumina microspheres were found to be 6.4 and 5.1 μm, respectively. Sterile microspheres and microsphere-coated plates were prepared by the method previously reported [5]. The obtained microspheres and microsphere-coated plates (20 μg/well) were subjected to the assays.

Cellular Assays. Normal human osteoblasts (NHOst) were purchased from BioWhitteker Inc. (Walkersville, MD). The cells were maintained using alpha minimum essential medium (Gibco) containing 20% fetal calf serum (FCS) in incubators (37°C, 5%-CO_2-95%-air, saturated humidity).

All assays were carried out using the medium supplemented with 10mM β-glycerophosphate. NHOst ($2x10^4$ cells/well/500 μl medium) were cultured on the microsphere-coated plates for estimating the effect of the microspheres from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NHOst were cultured with microsphere-containing medium (20 μg/500 μl medium) after they had adhered to the collagen-coated plates. The cell number ratio of NHOst cultured with microspheres was evaluated using the alamar Blue™ assay (BioSource International, Inc., Camarillo, CA), which incorporates an oxidation-reduction indicator based on the detection of metabolic activity, according to manufacturer's instruction.

The level of alkaline phosphatase (ALP) activity of the NHOst and the amounts of calcium deposited during a 7-day incubation were evaluated to estimate differentiation level of NHOst as previously reported [6]. In addition, RT-PCR was performed to detect the expression of osteocalcin mRNA in the NHOst (primers for human osteocalcin [7]; forward 5'CATGAGAGCCCTCACA3' and reverse 5'AGAGCGACACCCTAGAC3'; product size 307-bp).

Gap junctional intercellular communication (GJIC), which is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules [8], among NHOst co-cultured with microspheres were evaluated using FRAP assay as previously reported [9].

All data were expressed as the mean value ± the standard deviation (SD) or the standard error of means (SEM) of the obtained data as indicated in all figures and tables. The Fisher-Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

Results and Discussion

Figure 1 shows the effect of the diameter of pre-coated polystyrene microspheres on proliferation, the ALP activity of co-cultured NHOst cells, and the amounts of deposited calcium on the NHOst. To compare the effect of the microspheres on the ALP activities and the calcium amounts for each NHOst, the obtained data were standardized based on the cell number ratio co-cultured with the microspheres. As shown in figure, suppression on ALP activity of NHOst and the amounts of deposited calcium were observed when 0.1 μm and 5 μm microspheres were co-cultured. When the microspheres were added after cell adhesion, they did not show a significant inhibitory effect on the functions of NHOst (data not shown). By pre-coating of the microspheres on the bottom of the test plates, the area they occupied became larger as their diameter became smaller. This increase in the microsphere occupied area would affect many functions of the test cells, resulting in the inhibitory effect of the 0.1μm microspheres on the function of NHOst when the same quantity of microspheres was coated. On the other hand, the suppression of ALP activity of NHOst and calcium deposition by pre-coated 5 μm PS microspheres suggests that not only the area they occupied but also their size may cause the unique inhibitory activity of the 5 μm PS microspheres. It is well known that the size of a microsphere plays an important role in phagocytosis [10], although it is unclear that there is the same size dependence on phagocytosis by the NHOst as by macrophages. In addition, our previous study suggested that even fibroblasts were likely to phagocyte microspheres of a specific

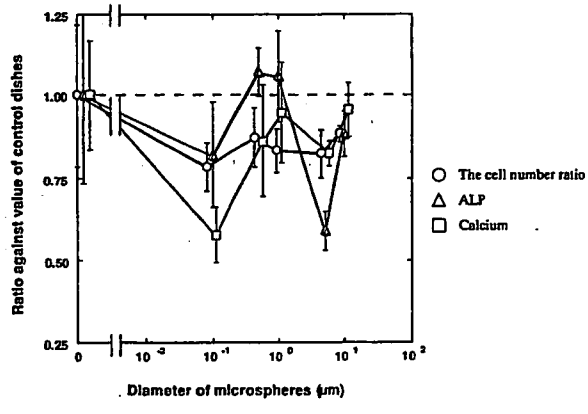

Figure 1. Effects of diameter of pre-coated PS on various functions of NHOst. Data are shown as the means±SD

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±5.5	88.2±2.2	92.2±1.3	82.4±2.8	83.0±2.3
Percent ALP activity (activity/proliferation)	100.0±4.7	79.2±5.6	72.7±3.6*	58.2±5.7*	73.8±6.0*
Percent deposited calcium (Calcium percent/proliferation)	100.0±3.7	97.3±4.2	82.3±3.7	90.3±7.8	163.3±18.5*(a

Data are shown as the mean value ± 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.

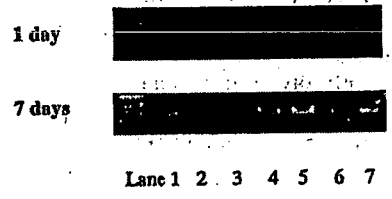

Figure 2. Expression of osteocalcin mRNA extracted from NHOst cultured on various microsphere pre-coated dish. Lane 1: Collagen-coated culture dish, 2: methanol-treated dish, 3: PS, 4: PE, 5: alumina, 6: un-sintered HAp, 7: sintered HAp.

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References

[1] J.A.Savio III, L.M.Overcamp and J.Black, Clin. Mater., **15,** 101 (1994)
[2] T.R.Green, J.Fisher, J.B.Matthews, M.H.Stone and E.Ingham, J. Biomed. Mater. Res. (Appl. Biomater.), **53,** 490 (2000)
[3] M.C.D.Trindade, D.J.Schurman, W.J.Maloney, S.B.Goodman and R.L.Smith, J. Biomed. Mater. Res., **51,** 360 (2000)
[4] C.Vermes et al., J. Bone. Miner. Res., **15,** 1756 (2000)
[5] R.Nakaoka, T.Tsuchiya, K.Sakaguchi and A.Nakamura, J. Biomed. Mater. Res., **57,** 279 (2001)
[6] M.Nagahata, R.Nakaoka, A.Teramoto, K.Abe and T.Tsuchiya, Biomaterials, **26,** 5138 (2005)
[7] M.M.Levy et al., Bone, **29,** 317 (2001)
[8] A.D.Maio, V.L.Vega and J.E.Contreras, J. Cell. Physiol., **191,** 269 (2002)
[9] R.Nakaoka and T.Tsuchiya, Mater. Trans., **43,** 3122 (2002)
[10] Y.Tabata and Y.Ikada, Adv. Polym. Sci., **94,** 107 (1990)
[11] T.Tsuchiya, J. Biomater. Sci. Polymer Edn., **11,** 947 (2000)
[12] H.J.Donahue, Z.Li, Z.Zhou and C.E.Yellowley, Am. J. Physiol. Cell Physiol., 278, C315 (2000)
[13] R.Nakaoka, S.Ahmed and T.Tsuchiya, J. Biomed. Mater. Res., in press
[14] K.Degroot, Biomaterials, **1,** 47 (1980)
[15] J.Chen, H.S.Shapiro and J.Sodek, J. Bone Miner. Res., **7,** 987 (1992)

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).

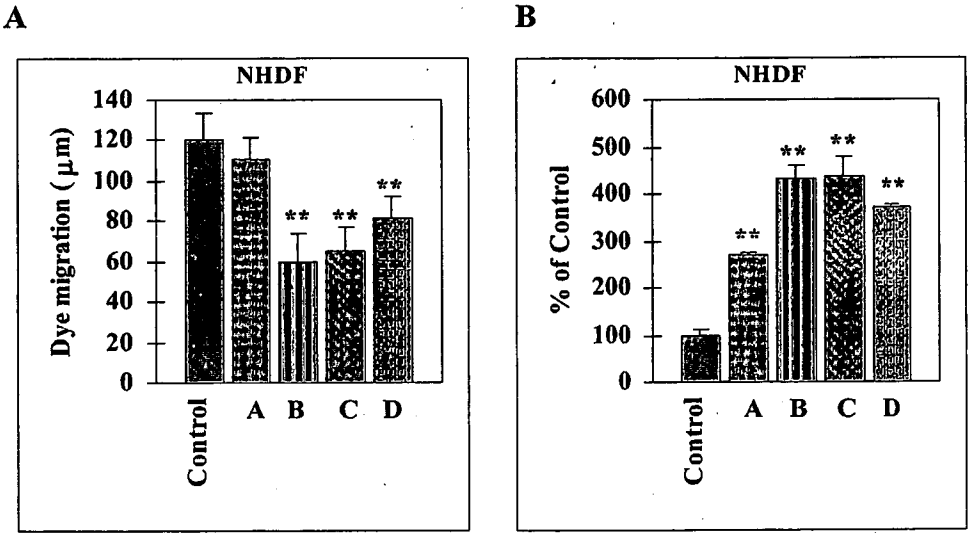

Figure 1. In A, Statistical analysis of SLDT assay and in B, cell proliferation of NHDF cells. ** p < 0.01.

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.

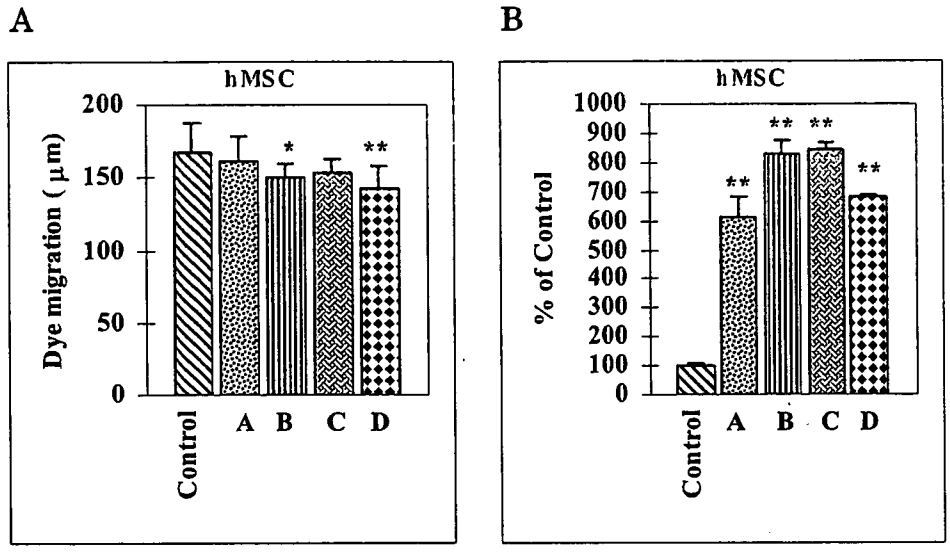

Figure 2. In A, Statistical analysis of SLDT assay and in B, cell proliferation of hMSCs. * $p < 0.05$, ** $p < 0.01$.

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.

5. REFERENCES

[1] Pittenger, M.F. *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells, *Science.* 284, 143-147.

[2] Giraux, J.L., Matou, S., Bros, A., Tapon-Bretaudiere, J., Letourneur, D. and Fischer, A.M. (1998) Modulation of human endothelial cell proliferation and migration by fucoidan and heparin, *Eur J Cell Biol.* 77, 352-359.

[3] Sweeney, E.A., Lortat-Jacob, H., Priestley, G.V., Nakamoto, B. and Papayannopoulou, T. (2002) Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells, *Blood.* 99, 44-51.

[4] Klaunig, J.E. and Ruch, R.J. (1990) Role of inhibition of intercellular communication in carcinogenesis, *Lab Invest.* 62, 135-146.

[5] Matsubara, T. *et al.* (2004) A new technique to expand human mesenchymal stem cells using basement membrane extracellular matrix, *Biochem Biophys Res Commun.* 313, 503-508.

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

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

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

1. INTRODUCTION

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

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

2. MATERIALS AND METHODS

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

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

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

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

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

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

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

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

3. RESULTS

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

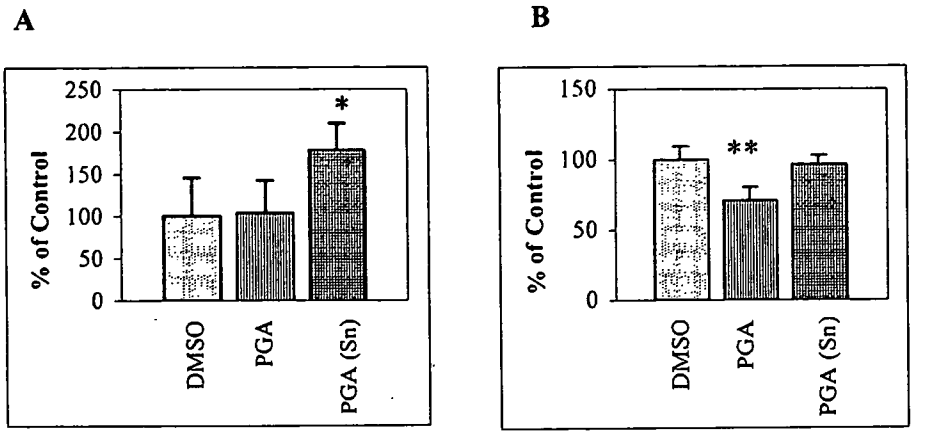


Figure 1. Cell proliferation (A) and cell differentiation (B) of human articular chondrocytes after 4 weeks culture period. * $p < 0.05$, ** $p < 0.01$.

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

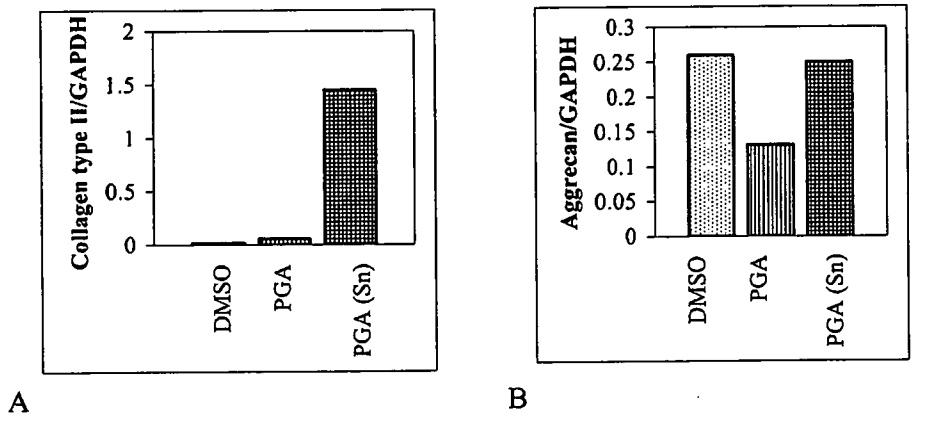
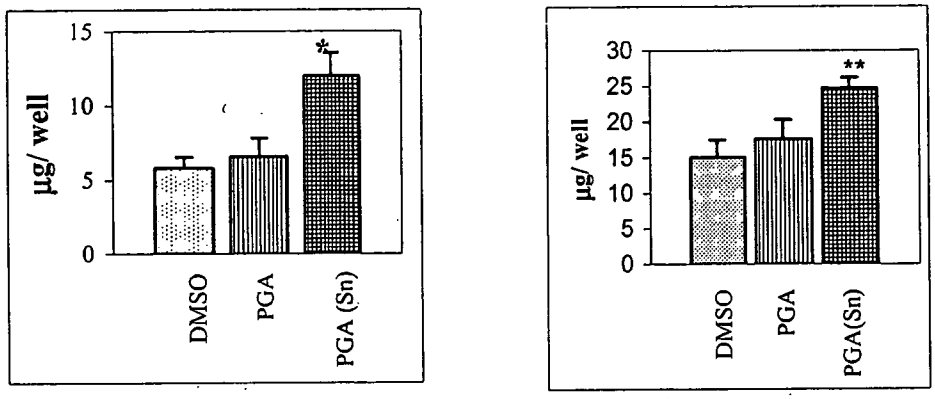


Figure 2. Expression of collagen type II gene (A) and aggrecan gene (B) in cultured chondrocytes, estimated by real time PCR method.

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



A B

Figure 3. Estimation of the amount of collagen type II protein (A) and total collagen (B) of human articular chondrocytes after 4 weeks of culture. ** $p < 0.01$.

4. DISCUSSION

Different tin compounds had already exhibited general cytotoxic effects on rabbit articular cartilage in monolayer culture [6], and Yamaguchi et al. suggested bone as the critical organ in inorganic tin toxicity in rats [7]. We evaluated the chondrogenic effect of HAC with PGA, synthesized with and without inorganic tin catalyst, in micromass culture system. Oral administration of certain tin compounds was reported to exert stimulatory effect on chondrocyte proliferation of rat [6]. Parallel with this event, proliferation assay of HAC with PGA (Sn) performed in our study also showed stimulatory effect on chondrocyte proliferation in micromass culture (Fig 1). But, PGA showed neither inhibition nor stimulation on the chondrocyte proliferation and thus inorganic tin as catalyst seemed to play a stimulatory role in HAC proliferation. In rat, oral administration of inorganic tin was reported to cause decrease in the proliferation of the chondrocytes accompanied by suppression of DNA synthesis with subsequent inhibition in collagen synthesis [8]. These references suggested a direct relation of inorganic tin in chondrocyte proliferation with the synthesis of collagen protein. In support of these suggestions, our results also showed enhancement of HAC proliferation, expression of collagen type II gene, and amounts of total collagen and collagen type II protein. There was a strong decrease in aggrecan gene expression in PGA compared with control. This study firstly to show the biological action of inorganic tin as catalyst in PGA on human articular chondrogenesis in a micromass culture system. We speculate that nature of tin compound, and also the route of application may play a key role in exhibiting various chondrogenic effects of this metallic compound. In

spite of different positive findings regarding human articular chondrogenesis, from the view points of safetyness we are considering inorganic tin catalyst is not appropriate to use for synthesis of biodegradable polymers in future clinical applications.

5. REFERENCES

[1] Chang, L.W. (1990) The neurotoxicology and pathology of organomercury, organolead, and organotin, *J. Toxicol. Sci.* 15, 125-151.

[2] De Mattos, J.C. *et al.* (2000) Damage induced by stannous chloride in plasmid DNA, *Toxicol. Lett.* 27, 159-163.

[3] Chao, J.S., Wei, L.Y., Huang, M.C., Liang, S.C. and Chen, H.H. (1999) Genotoxic effects of triphenyltin acetate and triphenyltin hydroxide on mammalian cells in vitro and in vivo, *Mutat. Res.* 21, 167-174.

[4] Chang, L.W. (1984) Hippocampal lesions induced by trimethyltin in the neonatal rat brain, *Neurotoxicology.* 5, 205-215.

[5] Brown, A.N., Kim, B.S., Alsberg, E. and Mooney, D.J. (2000) Combining Chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs, *Tissue Eng.* 6, 297-305.

[6] Webber, R.J., Dollins, S.C., Harris, M. and Hough, A.J. Jr. (1985) Effect of alkyltins on rabbit articular and growth-plate chondrocytes in monolayer culture, *J. Toxicol. Environ. Health.* 16, 229-242.

[7] Yamaguchi, M., Kitade, M. and Osaka, S. (1980) The oral administration of Stannous chloride to rats, *Toxicol. Lett.* 5, 275-278.

[8] Yamaguchi, M., Sugii, K. and Okada, S. (1982) Inhibition of collagen synthesis in the femur of rats orally administered stannous chloride, *J. Pharm. Dyn.* 5, 388-393.

INCREASE IN THE INSULIN SECRETION OF HIT-T15 CELLS:

Gap Junctional Intercellular Communications Enhanced by Hyaluronic Acid

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

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

1. INTRODUCTION

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

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

2. MATERIALS AND METHODS

2.1 Preparation of media and culture dishes

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

2.2 Cell culture

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

2.3 Measurement of cell viability

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

2.4 Measurement of insulin release

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

2.5 Scrape-loading and dye transfer (SLDT) assay

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

3. RESULTS AND DISCUSSION

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