GJIC relates to more or less production of the factors. Thus, MB cell differentiation may be affected by immobilized molecules through physicochemical feature of PE surface and recovery in the GJIC function of the mesenchymal cells. To prove this hypothesis about the specificity of the effects of immobilized molecule, it is indispensable to develop another experimental method with competitive chemicals binding to specific integrins.

Although collagen immobilization onto PE film increased differentiated foci of MB cells when cultured on the film, the number of the foci was comparable to that observed on a collagen-coated culture dish. As shown in Table I, however, proliferation of MB cells on the coated collagen is facilitated compared to that on the immobilized collagen. Therefore, there must be a difference in the effect of coated and immobilized collagen on the behavior of MB cells. As shown in Figure 2, the size and shape of the foci were different on the collagen-coated dish and collagen-immobilized PE. In addition, more nutrition was needed for MB cells on collagen-immobilized PE than for those on the collagen-coated dish, judging from the results shown in Table II, which suggests that MB cells on collagen-immobilized PE are more motile than those on the collagen-coated dish, resulting in their rearrangement into aggregated small foci (Figs. 2 and 3). Therefore, it is probable that the difference in MB cell behavior can be ascribed to a difference in molecular structure of coated collagen and immobilized collagen. Table II also indicated that the condition medium from collagen-immobilized PE had the potential to induce MB cell differentiation as well as that from the collagen-coated dish, although its cytotoxicity was higher. This suggests that MB cells on the immobilized PE produce unknown factors that improve their differentiation more than those on the coated dish. It has been reported that recovering GJIC function of dermal fibroblasts results in enhancement of growth factor production.[16] Therefore, it is probable that enhanced GJIC function of MB cells is induced by their aggregation. Although the amount of coated collagen on the dish is unknown, it is likely that immobilized collagen is suitable to prepare a surface for cell culture, biomaterials, scaffolds, and so on. To discuss different features of collagen-coated and collagen-immobilized surfaces for biomaterials more precisely, further studies, including an electrophysiological study of differentiated MB cells, are needed.

In conclusion, not only production of the factors but also direct interaction between the nerve cells and the mesenchymal cells may be responsible for the differentiation of MB cells. Moreover, it is probable that a surface characteristic of the biomaterial affects both the production and the interaction, as well as the GJIC function of cells on the surface. However, this study suggests that surface improvement of biomaterials for cell adherence is enough, in some cases, to maintain normal cell function and differentiation. Thus, a biomaterial surface should be carefully designed according to the purpose of the biomaterial.

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Enhancing effect of poly(L-lactide) on the differentiation of mouse osteoblast-like MC3T3-E1 cells

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Abstract

Poly(L-lactide) (PLLA) has bioabsorbability and biocompatibility, and it is used as biodegradable screws, pins and plates for internal bone fixation. The purpose of this study was to clarify the effects of low molecular weight (Mw) PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) for 2 weeks using the micromass culture. Both PLLAs did not affect the proliferation of MC3T3-E1 cells. However, the calcifications of MC3T3-E1 cells were stimulated with increasing the concentration of the PLLAs. Then PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k. Additionally, both PLLAs increased the alkaline phosphatase (ALP) activity and calcium content of MC3T3-E1 cells up to the similar level to the calcification. These results indicated that low Mw PLLA enhanced the differentiation of MC3T3-E1 cells with no effect on the proliferation. Moreover, it was suggested that the increase of the ALP activity was a key step to stimulate the calcification of MC3T3-E1 cells. The osteoconductivity of implanted PLLA would be based on the enhancing effect of low Mw PLLA on the differentiation of the osteoblasts.

Keywords: Poly(L-lactide); Osteoblast; MC3T3-E1 cell; Calcification; Differentiation; Micromass culture

1. Introduction

Poly(L-lactide) (PLLA) with a high molecular weight (Mw) is used as biodegradable screws, pins and plates for internal bone fixation in the orthopedics. Bos et al. reported that the mass loss of PLLA was observed after 26 weeks, and no acute or chronic inflammatory reaction to PLLA was observed until 143 weeks with exception of the early part implant period, by subcutaneous implantation into rats [1]. Otto et al. observed lamellar bone formation around the PLLA wire at 2 and 6 months after intramedullary implantation into rat tibiae [2]. Mainil-Varlet et al. also observed decreasing Mw of PLLA after 4 weeks and bone formation around the PLLA pin at 1 month after implantation into the cortex of sheep tibiae [3]. Thus, there have been many reports on the bioabsorbability and biocompatibility of PLLA.

Recently, there were reported that the lower change in the Mw of PLLA by heat treatment [4] and γ-ray irradiation [5] was responsible for enhancing the differentiation of mouse osteoblast-like MC3T3-E1 cells cultured on the PLLA. It was expected that the low Mw PLLA produced by degradation should enhance the differentiation of osteoblasts. Ikarashi et al. examined the response of MC3T3-E1 cells cultured on several PLLAs with different Mws. The alkaline phosphatase (ALP) activity increased when the cells were cultured on the PLLA with weight average Mw of 20,000 for 2 weeks, but not on the PLLA with Mw of 270,000 and 1,370,000. They also reported that the ALP activity increased when MC3T3-E1 cells were cultured with low Mw poly(DL-lactide) (PDLLA) for 2 weeks [6]. However, the proliferation and differentiation of MC3T3-E1 cells cultured with low Mw PLLA have not been clarified, and it is insufficient to discuss osteoblast differentiation only based on the ALP activity.

In the present study, MC3T3-E1 cells were cultured with low Mw PLLA using the micromass culture, and the differentiation of the cells was synthetically

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evaluated from the ALP activity and calcification. Thus, it was possible to clarify the effects of low Mw PLLA on the proliferation and differentiation of osteoblast-like MC3T3-E1 cells.

2. Materials and methods

2.1. Materials

PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The polydispersity indexes, which were calculated as the ratio of the weight average Mw to the number average Mw, of the PLLA-5k and PLLA-10k were, respectively 2.5 and 2.8, by gel permeation chromatography. The PLLAs were used without any refining.

PLLA-5k and PLLA-10k were, respectively, dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml, and sterilized by filtration through a 0.22 μm filter. The sterilized PLLA solutions were serially diluted with DMSO to give concentrations of 5, 10 and 25 mg/ml.

2.2. Cells

Mouse osteoblast-like MC3T3-E1 cells were obtained from RIKEN Cell Bank (Saitama, Japan). MC3T3-E1 cells were grown in alpha minimum essential medium (α-MEM) (Gibco Laboratories, Grand Island, New York, USA) supplemented with 20% fetal bovine serum (Intergen, Purchase, New York, USA), 100 μg/ml penicillin and 100 mU/ml streptomycin in a 37°C humidified atmosphere of 5% CO_2. The cells were passaged with 0.05% trypsin and 0.1% ethylenediaminetetraacetic acid tetrasodium salts solution (Gibco Laboratories).

2.3. Micromass culture

Cell suspensions were prepared in the culture medium and adjusted to give 2×10^6 cells/ml. A 20 μl aliquot of the cell suspensions was delivered into each well of type I collagen coated 24-well plate (Iwaki Glass, Tokyo, Japan). After the spot-like cells were attached on the well, 1 ml of the culture medium containing 10 mM disodium β-glycerophosphate (β-GP) (Sigma Chemical Co., St. Louis, MO, USA) and 1 μl of the serially diluted PLLA solution was added. As a control, 1 μl of DMSO was added to the culture medium instead of the PLLA solution. The culture medium containing each chemical at the same concentration was changed three times a week, and the cells were cultured for 2 weeks.

2.4. Proliferation assay

The proliferation of MC3T3-E1 cells was determined by using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co., Tokyo, Japan). The cell cultures were exchanged with the culture medium containing 0.1 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, 4 μM 1-methoxy-5-methylphenazinium methylsulfate and 3 mM NaCl, and were incubated for 2 h. The absorbance of the medium was read at 450 nm (reference at 600 nm) with a plate reader (μQUANT, Bio-Tek Instruments, Inc., Winooski, VT, USA). It has been proven that the absorbance and cell population show the linear relationship.

2.5. Calcification assay

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution. The alizarin red S solution was freshly made; 0.1 ml of 28% ammonia solution in 100 ml distilled water was added to 1 g of alizarin red S in 100 ml distilled water to make it pH 6.36–6.40. After the proliferation was determined, the cell cultures were washed three times with Dulbecco's phosphate-buffered saline without calcium and magnesium salts (PBS(−)) and fixed by the addition of 10% formalin dissolved in PBS(−) solution. After fixing, the cell cultures were washed five times with distilled water and stained by alizarin red S solution for 5 min. The transmission digital images of alizarin red S stained cultures were obtained with a color image scanner (GT-9500WIN, SEIKO EPSON Co., Nagano, Japan) with a transparency unit (GT95FLU, SEIKO EPSON Co.), and then, the alizarin red S stained areas were measured using an image processing and analysis software, Scion Image (Scion Co., Frederick, MD, USA).

2.6. Preparation of cell lysates

The ALP activity and calcium content of MC3T3-E1 cells cultured with low Mw PLLA for 2 weeks were measured using the cell lysates [7]. The cell cultures were washed twice with PBS(−). The cells were recovered by trypsinization and washed twice with PBS(−) by centrifugation at 1000 rpm for 2 min. The residues were resuspended in 1 ml of 0.2% Nonidet P-40 and sonicated in an ice bath for 2 min using an ultrasonic processor (VC-50T, Sonic & Materials Inc., Danbury, CT, USA). The cell lysates were stored frozen at −20°C until measurement of the ALP activity and calcium content.

2.7. ALP activity

The ALP activity of cell lysates was measured according to the method of Ikarashi et al. [4]. The same

quantity of 2 mM $MgCl_2$ in 0.1 M carbonate buffer (pH 10.2) and 20 mM *p*-nitrophenylphosphate were mixed as the substrate solution, and then, this substrate solution was pre-incubated at 37°C. Twenty microliters of the cell lysates was incubated with 1 ml of the substrate solution at 37°C for 30 min. The enzymatic reaction was stopped by adding 2 ml of 0.25 N NaOH, and the absorbance of *p*-nitrophenol liberated was read at 410 nm. The calibration curve of ALP activity was made by the standard solutions that diluted calf intestine ALP (Boehringer Mannheim GmbH, Germany) at the various concentrations. Total protein content of cell lysates was measured by the method of Lowry et al. [8] with minor modification using bovine serum albumin (Wako Pure Chemical Industries, Ltd.) as a reference standard [5]. The ALP activity of cell lysate was normalized for total protein content of the cell lysate.

2.8. Calcium content

The calcium content of cell lysates was determined by using a diagnostic kit, Calcium C (Wako Pure Chemical Industries, Ltd.). The same quantity of the cell lysates and 1 N HCl were mixed, and decalcified for 15 h at room temperature [9]. Ten microliters of the decalcifying solution and 1.0 ml of 0.88 M monoethanolamine buffer (pH 11.0) were mixed, and 100 µl of 0.63 mM *o*-cresolphthalein complexon and 69 mM 8-hydroxyquinoline was added. After 15 min at room temperature, the absorbance of the reaction solution was read at 570 nm. The standard calcium solutions of various concentrations were also operated by the same manner in order to make the calibration curve.

2.9. Statistical analysis

All measured values were collected in four sets and expressed in means ± standard deviation (SD). Differences among the groups were evaluated with one-way or two-way analysis of variance (ANOVA). When significant differences among the groups were found, Tukey–Kramer test was applied for multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Proliferation

Mouse osteoblast-like MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k or PLLA-10k for 2 weeks using the micromass culture. In microscopic observation, the proliferations of MC3T3-E1 cells cultured with PLLA-5k and PLLA-10k were almost the same as that of the control group during the culture period. Fig. 1 shows the effects of PLLA-5k and PLLA-10k on the proliferation of MC3T3-E1 cells. There was no significant difference between the proliferation of the cells cultured with and without PLLA-5k ($P = 0.7537$) and PLLA-10k ($P = 0.7521$) by one-way ANOVA. PLLA-5k and PLLA-10k up to 50 µg/ml did not affect the proliferation of MC3T3-E1 cells.

3.2. Calcification

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution (Fig. 2). Because alizarin red S combines with the calcium and forms the

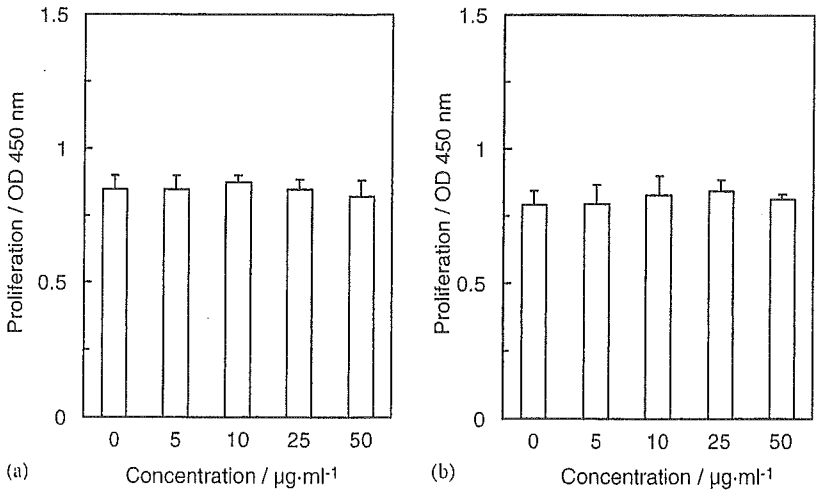

Fig. 1. The effect of PLLA on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The proliferation of MC3T3-E1 cells cultured with the PLLA was determined using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co.). Values are means ± SD for four dishes. Significant difference at $P < 0.05$ was not found among the groups by one-way ANOVA.

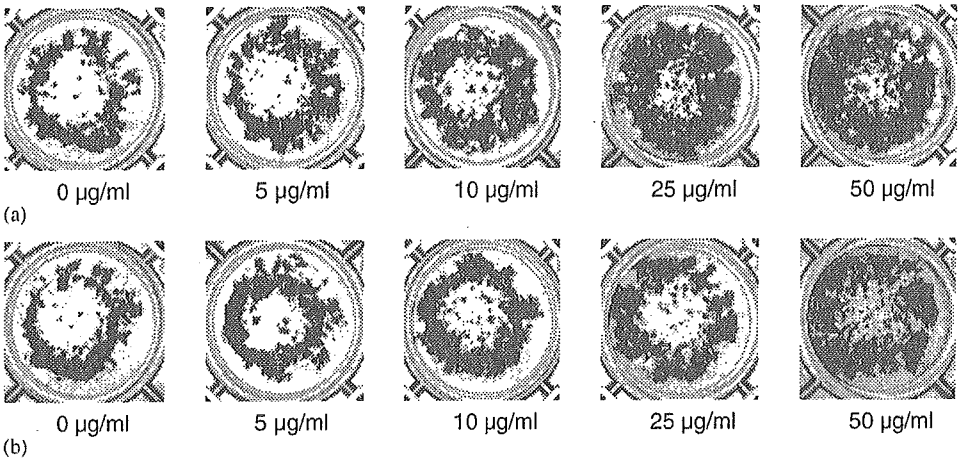

Fig. 2. The appearance of alizarin red S staining of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The culture of MC3T3-E1 cells was stained by alizarin red S solution.

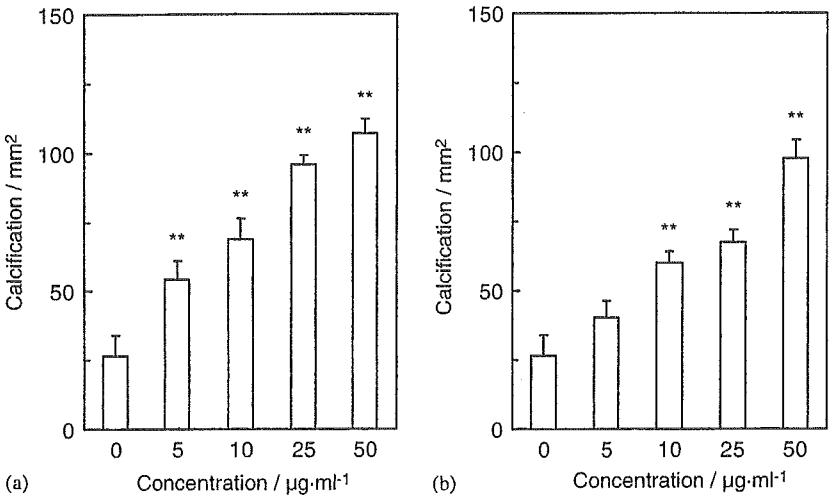

Fig. 3. The effect of PLLA on the calcification of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcification of MC3T3-E1 cells cultured with the PLLA was determined by measuring the alizarin red S stained areas using an image processing and analysis software, Scion Image (Scion Co.). Values are means ± SD for four dishes. Significant difference compared with control (without PLLA) at $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

lac of poor solubility, the calcification parts in cell cultures are stained dark-red. The calcification parts clearly increased with increasing the concentrations of PLLA-5k and PLLA-10k. The total area of the calcification parts in the well was measured to determine the extent of the calcification of cell cultures. Fig. 3 shows the effect of PLLA-5k and PLLA-10k on the calcification of MC3T3-E1 cells. The calcification of MC3T3-E1 cells cultured with PLLA-5k increased 2.1-fold at 5 μg/ml and increased 4-fold at 50 μg/ml (Fig. 3(a)), and then the calcifications of the cells were significantly increased with increasing the concentrations of the PLLA-5k by one-way ANOVA ($P<0.0001$). On the other hand, the calcification of MC3T3-E1 cells cultured with PLLA-10k increased 1.5- and 3.7-fold at 5 and 50 μg/ml, respectively (Fig. 3(b)), and then the calcifications of the cells were also significantly increased with increasing the concentrations of the PLLA-10k by one-way ANOVA ($P<0.0001$). PLLA-5k and PLLA-10k stimulated the differentiation of MC3T3-E1 cells cultured with the PLLAs, dose-dependently ($A = 11.86\ \mathrm{sqrt}(C_{5k}) + 28.99$, $r = 0.9880$, $P = 0.0016$, A: total area of calcification parts (mm^2), C_{5k}: concentration of PLLA-5k (μg/ml); $A = 9.93\ \mathrm{sqrt}(C_{10k}) + 23.73$, $r = 0.9807$, $P = 0.0032$, C_{10k}: concentration of PLLA-10k (μg/ml)). Moreover, when it was compared at the weight concentration of PLLA, PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k significantly ($P = 0.0075$ by two-way ANOVA).

3.3. ALP activity and calcium content

ALP is a representative enzyme of osteoblastic differentiation, and then ALP activity was determined

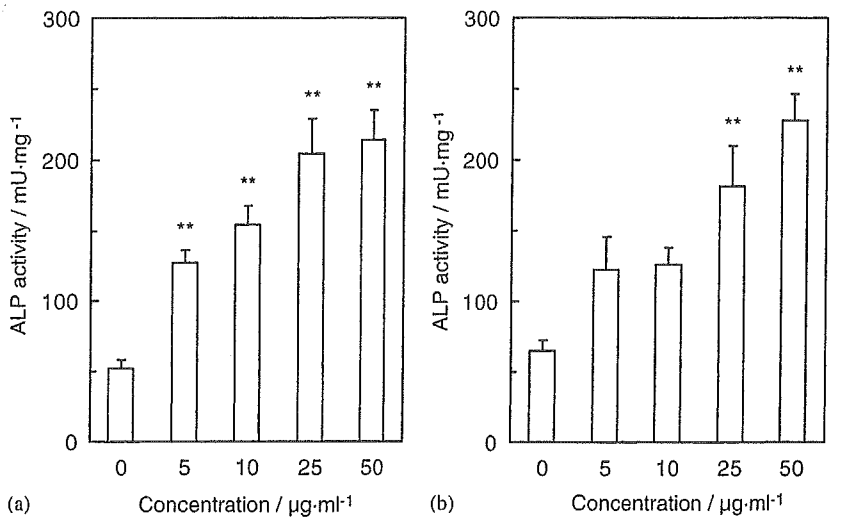

Fig. 4. The ALP activity of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The ALP activity of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using *p*-nitrophenylphosphate as a substrate. Values are means±SD for four dishes. Significant difference compared with control (without PLLA) at $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

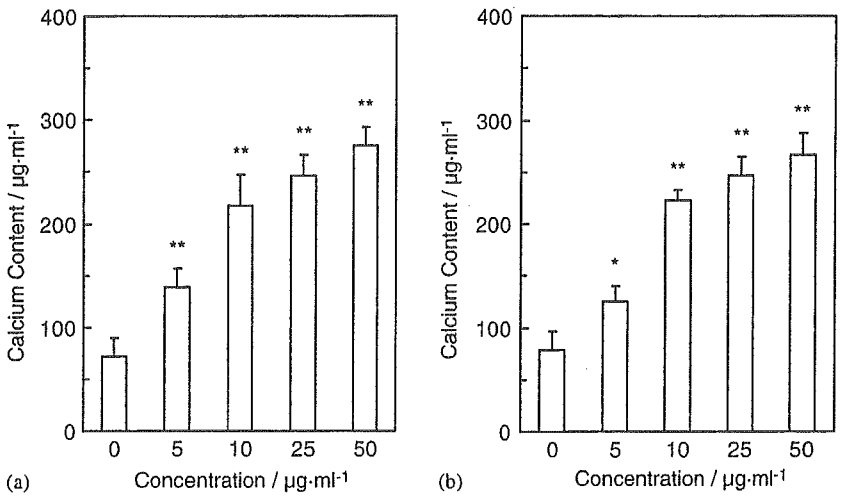

Fig. 5. The calcium content of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcium content of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using a diagnostic kit, Calcium C (Wako Pure Chemical Industries, Ltd.). Values are means±SD for four dishes. Significant difference compared with control (without PLLA) at $^{*}P<0.05$ or $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

as an indicator of osteoblastic differentiation of MC3T3-E1 cells cultured with PLLA-5k or PLLA-10k. Fig. 4 shows the effects of PLLA-5k and PLLA-10k on the ALP activity of MC3T3-E1 cells. ALP activities of the cells cultured with the PLLA-5k ($P<0.0001$) and PLLA-10k ($P<0.0001$) significantly increased with increasing the concentrations of the PLLAs by one-way ANOVA.

Calcification is also an important indicator of osteoblastic differentiation, and the calcium content was determined in order to verify the result of the above calcification assay. Fig. 5 shows the effects of PLLA-5k and PLLA-10k on the calcium content of MC3T3-E1 cells. Calcium contents of the cells cultured with the PLLA-5k ($P<0.0001$) and PLLA-10k ($P<0.0001$) significantly increased with increasing the concentrations of the PLLAs by one-way ANOVA. The results of calcium content completely agreed with the results of calcification assay of the cells cultured with PLLA-5k and PLLA-10k.

4. Discussion

The implanted PLLA nonenzymatically hydrolyzes in vivo, and the Mw of the PLLA decreases. Bergsma et al. determined the PLLA particles with number average molecular weight (Mn) of 5600 and 5400 at 3.3 and 5.7

Table 1
The comparison among the cell responses of MC3T3-E1 cells cultured with PLLA

Sample	Concentration (μg/ml)	Proliferation (%)	Calcification (%)	ALP activity (%)	Calcium content (%)
PLLA-5k	0	100	100	100	100
	5	100	205	241	193
	10	103	260	293	302
	25	100	361	388	343
	50	97	404	407	384
PLLA-10k	0	100	100	100	100
	5	100	152	188	161
	10	105	227	193	285
	25	106	254	277	316
	50	103	369	349	341

The ratios of the proliferation, calcification, ALP activity and calcium content of MC3T3-E1 cells cultured with PLLA-5k or PLLA-10 to the control (without PLLA) were calculated.

years, respectively, after the implantation of PLLA plates or screws (Mn of 760,000) into patients [10]. Bos et al. reported that the Mw of PLLA rapidly decreased in the first 12 weeks, but the mass loss of the PLLA continued after 78 weeks, after the subcutaneous implantation of PLLA (viscosity average molecular weight (Mv) of 900,000) into rats [1]. Mainil-Varlet et al. also observed that the weight average Mw of PLLA implanted into the cortex of sheep tibiae decreased from 61,900 to 42,600 at 4 weeks, to 20,800 at 12 weeks and to 9206 at 52 weeks [3]. Kinoshita et al. reported that the degradation and absorption of PLLA mesh (Mw of 200,000) implanted subcutaneously in the back and subperiosteally at the calvaria of rats continued after 30 months, and then many macrophages appeared in the circumferential tissue of hydrolyzed PLLA particles [11]. The purpose of the present study was to clarify the effects of low Mw PLLA, which would be produced by degradation, on the proliferation and differentiation of mouse osteoblasts in vitro.

In the present experiment, we added 10 mM β-GP to the α-MEM medium, and MC3T3-E1 cells were micromass cultured with low Mw PLLA. β-GP displays synergistic action with ascorbic acid to further stimulate collagen accumulation and ALP activity in osteoblasts, and mature osteoblasts require β-GP for mineralization [12]. Moreover, Quarles et al. also reported that MC3T3-E1 cells actively proliferated before attaining confluence, but failed to express ALP activity and did not accumulate mineralized extracellular collagenous matrix at this stage. After the cultures underwent growth arrest owing to the attainment of confluence, ALP activity and mineralized extracellular collagenous matrix were expressed [12]. The cell density of the micromass culture is extremely high, and the situation of the micromass culture is similar to state of confluence from initial stage of culture. Ikarashi et al. cultured MC3T3-E1 cells in the α-MEM without β-GP on the monolayer, and observed the calcification of MC3T3-E1 cells by alizarin red S staining after the culture as long as 4 weeks [6]. However, we cultured MC3T3-E1 cells in the α-MEM with β-GP using the micromass culture, and succeeded in the detection of the calcification of MC3T3-E1 cells in the short period of 2 weeks (Fig. 2).

The osteogenesis is the most important phenotype of the osteoblasts and it has been confirmed that MC3T3-E1 cells form the calcified bone tissue in vitro [13,14]. Therefore, the calcification was an index of the final differentiation of MC3T3-E1 cells for the present experiment. PLLA-5k and PLLA-10k did not affect the proliferation of MC3T3-E1 cells cultured with the PLLAs (Fig. 1). On the other hand, the calcification of MC3T3-E1 cells cultured with the PLLAs increased with increasing the concentration of the PLLAs dose-dependently (Fig. 3). These results indicate that the low Mw PLLA stimulated the differentiation of MC3T3-E1 cells cultured with the PLLA with no effect on the proliferation. Ikarashi et al. also observed that the poly(DL-lactide) (PDLLA) with weight average Mw of 5000 and 10,000 did not affect the proliferation, but remarkably increased the calcification of MC3T3-E1 cells [6].

The cell responses of MC3T3-E1 cells cultured with the PLLAs were compared (Table 1). As described above, the PLLAs did not effect on the proliferation of MC3T3-E1 cells, but the PLLAs stimulated the calcification of MC3T3-E1 cells. The calcification of MC3T3-E1 cells cultured with 50 μg/ml of PLLA-5k and PLLA-10k increased approximately 4- and 3.7-fold, respectively. ALP activity and calcium content of MC3T3-E1 cells cultured with the PLLAs also increased to the similar level to the calcification. Thompson and Puleo observed that the ALP activity, osteocalcin content and calcium amount of bone marrow stromal cells greatly rose in the later stage of culture. They indicated that the osteoprogenitor cells first differentiated into immatute osteoblasts characterized by the expression of ALP and then into mature osteoblasts characterized by the

expression of osteocalcin and calcification [15]. The increase rates of ALP activity and calcification of MC3T3-E1 cells cultured with the PLLAs were almost equal in the present results. As the ALP activity increasing during the process of culture with PLLAs, the calcification of MC3T3-E1 cells would be stimulated. Otto et al. reported when mouse primary osteoblasts were cultured with the PLLA (Mv of 21,500) wire for 48 h, DNA content did not change, but ALP activity increased by 28% [16]. Ikarashi et al. also observed that the ALP activity of MC3T3-E1 cells remarkably increased with no effect on the proliferation, when the cells were cultured on the PLLA (weight average Mw of 20,000) and with the PDLLA for 2 weeks [6]. Our results corresponded to their results.

Ikarashi et al. reported that heat treatment decreased the Mw of PLLA, and then the heat treated PLLA did not effect the proliferation, but increased the differentiation of MC3T3-E1 cells cultured on the PLLA [4]. Isama et al. also reported that the Mw of PLLA was decreased by γ-irradiation, and then the irradiated PLLA increased the differentiation of MC3T3-E1 cells cultured on the PLLA with no effect the proliferation [5]. They describe that lower change in the Mw of PLLA would be responsible for enhancing the differentiation of MC3T3-E1 cells cultured on the heat irradiated or γ-irradiated PLLA. The present result that the low Mw PLLA enhanced the differentiation of MC3T3-E1 cells strongly supports their proposal.

We examined the effects of the low Mw PLLA only in the mouse osteoblat-like MC3T3-E1 cells. The MC3T3-E1 cells are the most widely used for the research of the bone formation. However, culture osteoblasts show various phenotypes according to cell lines [13]. It would be required that further studies to analyze the effect of PLLA on other osteoblasts, such as human osteoblasts if considering clinical use of PLLA.

In conclusion, the effects of low Mw PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells were investigated. The PLLA did not affect the proliferation of MC3T3-E1 cells. However, the ALP activity and calcification of MC3T3-E1 cells increased with increasing the concentration of the PLLA dose-dependently. These results indicated that the low Mw PLLA enhances the differentiation of MC3T3-E1 cells. Moreover, the increase rates of ALP activity and calcification of MC3T3-E1 cells cultured with the PLLA were almost equal. The increase of the ALP activity would be a critical step to stimulate the calcification of MC3T3-E1 cells. The present findings show that the osteoconductivity of implanted PLLA is based on the enhancing effect of low Mw PLLA on the differentiation of osteoblasts.

Acknowledgements

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DIFFERENT EXPRESSION OF GAP JUNCTIONAL PROTEIN CONNEXIN43 IN TWO STRAINS OF MICE AFTER ONE-MONTH IMPLANTATION OF POLY-L-LACTIC ACID

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Abstract. The implantation of a biomaterial often induces host inflammatory responses. Some adverse effects by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments. PLLA produced tumorigenicity in rats after long-term implantation. The purpose of this study was to determine the in vitro effect of PLAO3 (high-molecular weights of PLLA) and PU8 (PTMO/MDI/BD) on the function of the normal human dermal fibroblasts (NHDF) and the in vivo effect of PLAO3 on the function of the cells originated from the subcutaneous tissue in the two female mouse strains, BALB/cJ and SJL/J. The results with Scrape-loading and dye transfer (SLDT) assay, Western Blot and RT-PCR analysis clearly demonstrated that gap-junctional intercellular communication (GJIC) and the expression of Cx43 were significantly suppressed in PLAO3-implanted group of BALB/cJ mice in compared to the control mice. While, no significant difference was found in GJIC and the expression of mRNA level but a little bit difference was observed in the Cx43 protein expression between the SJL/J implanted and the control mice. We considered that the PLAO3 suppressed irreversibly gap junctional protein connexin43 at the earlier stage after implantation and the suppression of connexin43 gene-expression might play a vital role in the inhibition of GJIC and thus promotes the tumorigenesis.

Keywords: Poly-L-lactic acid, GJIC, Connexin43.

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1. INTRODUCTION

Some adverse effects caused by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments [1]. PLLA produced tumorigenicity in rats after long-term implantation. PUs were also used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, and good abrasion resistance. However, different kinds of PUs induced various tumor incidences in rats [2]. All tumors have been generally viewed as the outcome of disruption of the homeostatic regulation of cellular ability to response to extra-cellular signals, which trigger intra-cellular signal transduction abnormally [3]. We have hypothesized that the different tumorigenic potentials of PLLA and PUs are caused mainly by the different tumor-promoting activities of these biomaterials. In the present study, we investigated the effect of PLAO3 (a high-molecular weight PLLA) and PU8 on the normal human dermal fibroblast (NHDF). Our present results showed that the PLAO3 inhibited GJIC, whereas PU8 did not inhibit GJIC after 17 days culture on these materials. These findings inspired us to investigate the role of PLAO3 on the subcutaneous tissue of the two different responder strains of BALB/cJ and SJL/J mice.

2. MATERIALS AND METHODS

2.1. ***NHDF 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.

2.2. ***Animals:*** Five-week-old female BALB/cJ and SJL/J mice were obtained from Charles River (Japan).

2.3. ***Implantation of PLAO3:*** PLAO3 (20 X 10 X 1 mm) was obtained from Shimadzu Co. Ltd., and sterilized by ethylene oxide gas prior to use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. A dorsal incision of approximately 2 cm was made, opposite sites from the incision a subcutaneous pocket was formed by blunt dissection, and one piece of PLAO3 was placed in the pocket. The incision was closed with silk thread. In both strains, Sham's operation group served as controls. After 30 days, mice were sacrificed and subcutaneous tissues were obtained for subsequent culture.

2.4. ***Cell culture of subcutaneous tissues:*** The subcutaneous tissues were maintained in

minimum essential medium (MEM) supplemented with 10% FBS in a 5 % CO_2 atmosphere at 37°C. Cells were collected by trypsinization after adequate growth.

2.5. ***Giemsa staining:*** When cells reached confluence in tissue culture dishes, cells were fixed and stained with giemsa solution. Cells morphology was determined under an inverted light microscope.

2.6. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.05% 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.

2.7. ***Western Blot analysis:*** Cells were lysed directly in 100 μl of lysis buffer (50 mM Tris-Hcl, pH 6.8, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). Equivalent protein samples were then prepared in 7.5 % SDS-PAGE sample buffer containing 2-ME and loaded on 7% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Cx43 protein was detected by anti-Cx43 polyclonal antibodies and ECL system.

2.8. ***RT-PCR analysis:*** Total cellular RNA was isolated from cultured cells in Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by reverse transcript (RT) using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech). Amplification was performed in a volume of 25 μl containing 1 μl of cDNA, 10 pmol of each primer, 0.625 unit of *Taq* polymerase (Promega, Madison, WI, USA) and 0.2 mM of each deoxynucleotide triphosphate. The amplified product was electrophoresis using 1.5% agarose gel and visualized with SYBR Green. GAPDH gene was amplified as internal control.

3. RESULTS

NHDF cells: Giemsa staining showed that the NHDF cell cultures predominantly formed a uniform monolayer of cells. All cultures maintained the elongated shape of NHDF cells. There was no difference in morphology among the control, PLAO3 and PU8. In SLDT, the GJIC was significantly inhibited in PLAO3-exposed NHDF cells in

compared to the controls. On the contrary, no difference was observed between the PU8 implanted and the control groups.

Mouse cells: Inverted light microscopy and Giemsa staining showed that the mouse cells in cultures formed a cis-cross pattern and caused decreased contact inhibition in BALB/cJ control group (Figure 1A). On the other hand, in SJL/J control group, cells were parallel and maintained the contact inhibition (Figure 1C). All cells in the implanted groups of both the strains, showed cis-cross pattern and the cells were piled up in BALB/cJ group more than in SJL/J group (Figure 1B and 1D).

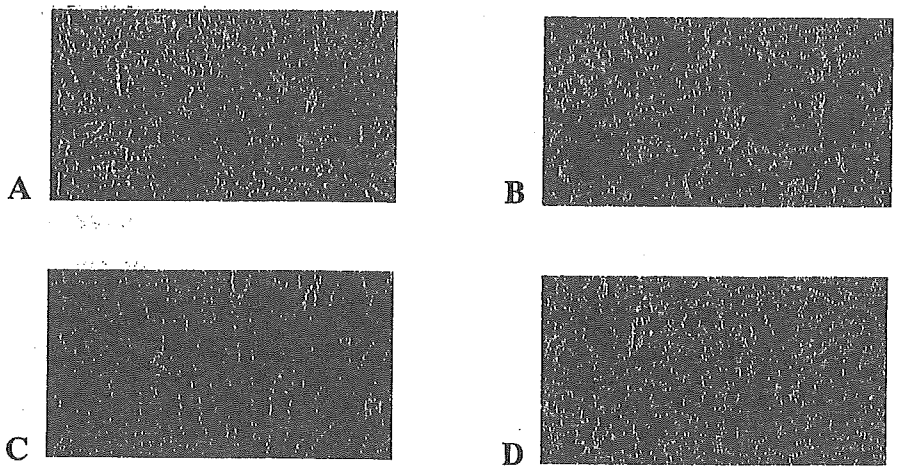

Figure 1 **Mice cells morphology. A: BAJB/cJ control, B: BALB/cJ implanted, C: SJL/J control and D: SJL/J implanted.**

In SLDT, the GJIC was significantly inhibited in PLAO3-implanted BALB/cJ cells in compared to BALB/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and its controls. To clarify the cause, we also examined the mRNA and protein expression levels of connexin43 gene and found that the mRNA and protein expression were suppressed in PLAO3-implanted BALA/cJ mice in compared to BALA/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and SJL/J controls.

4. DISCUSSION

Many factors, that caused tumorigenesis were known, we especially paid attention to the inhibition of the GJIC in the PLAO3-exposed cells. PLAO3 is a widely used

biomaterial for medical and surgical implants. Gap junctions are transmembrane channels that allow the cell-cell transfer of small molecules and are composed of protein subunits known as connexin; at least 19 connexins exist and they are expressed in various kinds of tissues of rodents. Several tumor promoters have been shown to inhibit GJIC by phosphorylation modification of connexin proteins. Connexins are essential proteins to maintain the gap junctional channel [4]. To understand the mechanisms of tumorigenesis induced by PLAO3, we paid attention to the inhibitory effects on GJIC. GJIC is important for normal differentiation of the cells such as neurons and osteoblasts. In the present study, the GJIC was inhibited in PLAO3-exposed NHDF and -implanted BALB/cJ mouse cells. This perturbed gap junction is most likely to play the major role in the PLAO3-induced tumorigenesis. Our results also showed that the mRNA and protein expression of connexin43 gene were suppressed in PLAO3-implanted BALB/cJ mice. Together with these results, we speculated that the inhibitory effect of PLAO3 on GJIC might be due to the alteration in the connexin43 protein. The post-translational modification and decrease in the connexin43 protein has been shown to be involved with impaired GJIC and could be associated in tumorigenesis mechanism. All experiments will be further analyzed at 6 and 12 months after PLAO3 implantation and these experimental data will give us the basic information that are useful for understanding the adverse event induced by medical and surgical implants.

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EVALUATION OF THE CORNEA CELLS AFFECTED BY MULTI-PURPOSE SOLUTIONS FOR CONTACT-LENS

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Abstract

We studied the influence of multi-purpose solutions (MPS) on gap-junctional intercellular communication (GJIC) of cornea cells and its cell toxicity such as cell metabolism and proliferation. This study evaluates long-term safety of three types of multi-purpose solutions (MPS) in the cornea cells. Three types of MPS were investigated using scrape-loading dye transfer (SLDT) method to measure GJIC. Cell toxicity was evaluated for cell viability according to colony method and USP elution method (MEM Elution Test). In the SLDT method, one type solution was significantly showed an inhibitory effect on the GJIC in cornea cells but not the other type solutions. Comparative cytotoxicity potentials of three types of MPS also indicated similar tendency in the result of SLDT method. These results suggest that the evaluation methods are considered to be effective to confirm the safety of MPS.

Key words: multi-purpose solution, contact lense, gap-junctional intercellular communication, cytotoxicity,

1. Introduction

Recently, various multi-purpose solutions (MPS) have been introduced to the market. Lens-cleaning products and surfactants also are common component in many types of MPS [1]. These components are the most important factor in safety of cornea cells for a

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long-term use. Spanakis *et al.* reported that cultured corneal fibroblasts and myofibroblasts have functional gap junctions to maintain intercellular communication with themselves and with nonactivated keratocytes [2]. To examine gap junction dependent intercellular communication (GJIC), a fluorescent dye transfer protocol based on the scrape-loading and dye transfer (SLDT) method developed by El-fouly *et al.* [3,4,5] was used for cultured monolayers. Gap junction channels play important roles in the maintenance of the stratified structure of the corneal cells [6,7]. Gong *et al.* reported the importance of gap junctions in maintaining normal lens transparency by providing a cell-cell signaling pathway [8]. We described a method for determining the inhibitory effects of MPS on the GJIC along with its cytotoxic effects. The method provides a means for the direct exposure of cornea cells to MPS. Cytotoxicity was indicated by significant increases in the number of dead cells relative to controls. In this study, three commercial MPS containing a variety of preserving/disinfecting agents were examined. The results indicate that this method is useful for measuring the safety of a multipurpose solution for contact lenses.

2. Materials and Methods

Cell culture: Human cornea cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO BRL) and antibiotics [penicillin (100 unit/ml)-streptomycin (100 μg/ml)]. Human cornea cells in DMEM-10% FCS medium were maintained in a humidified 5% CO_2 incubator at 37 °C. The cells were allowed to form a fully confluent monolayer.

Scrape-loading and dye transfer (SLDT) analysis: Human cornea parenchymal cells were incorporated at very high densities into the dish and allowed to form a fully confluent monolayer. The cell-monolayer was rinsed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)] before the addition of the fluorescent dye (Lucifer yellow : MW 457.2). The cell monolayer was scraped using a surgical blade and loaded with 0.1% Lucifer yellow solution. The dye solution was left with the cells, and they were incubated at 37 °C for 5 min in a humidified atmosphere containing 5% CO_2 and 95% air. The dye solution was discarded from 35 mm^2 plastic Petri dishes, and the dishes were washed three times with PBS(+) solution to remove detached cells and background fluorescence. The distance of dye migration

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). Measurement was carried out within 10 min after dye-loading.

MEM elution test: For measurement of cell toxicity, 5 x 10^4 cells seeded into 12 well plastic dishes. After 1 day, 0, 3.15, 6.25, 12.5, 25, 50, 100% volumes of multi-purpose solutions (MPS) in the media were applied to each well. The MPS-treated dishes were kept at 37 °C for 5 min in a humidified atmosphere containing 5% CO_2 and 95% air. After 3 days of MPS treatment, the extent of cell toxicity was measured by alamarBlue™ assay.

3. Results and Discussion

The GJIC functions of cornea cells were affected by MPS. Assessment of GJIC function was performed by SLDT assay using Lucifer yellow. Figure 1 shows fluorescent images of cells exposed to three types of MPS. The images show that Lucifer yellow diffuses through gap junctions from loaded cells to neighboring cells. The profile of percentages of dye migration in cornea cells is shown in Fig. 2. In the presence of MPS-A, dye transfer length has no significant change in comparison with the control (Fig. 2). While, the dye transfer extents of the cells treated with MPS-B and MPS-C were lower than that of the control. Decrease rate of dye transfer of MPS-B and MPS-C to the control is about 30% (Fig. 2). Therefore, these data indicated that cornea cells cultured with the MPS-B and MPS-C were induced GJIC down-regulation. These results suggest that the components of the MPS-B and MPS-C might cause the GJIC down-regulation.

Cell toxicity was evaluated for the assessment of the cell viability according to colony method and USP elution method. Figure 3 shows the estimation result of the cell toxicity performed by USP elution test. In all samples tested, the cell toxicities were higher than in saline buffer samples as the control. Thus, cell toxicities of all samples highly increased between 25% and 50% of MPS concentrations in the cell culture medium. Especially, the cell toxicity of MPS-B indicated high level in the colony method (data not shown). Based on these results, our results indicated that the conditions of MPS concentrations and MPS components are closely associated with safety of cornea cells, specifically with the expression of gap junction channels. Further investigations are required to clarify the cause of the inhibitory action on the GJIC and its *in vivo* adverse effects.

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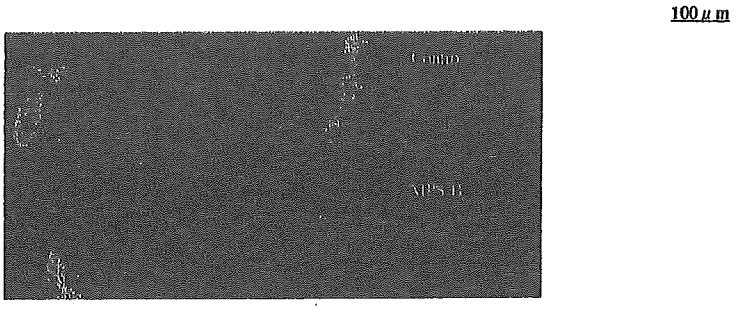

LY transfer after 2 hr incubation using various samples (20%)

Fig.1 Appearace of fluorescence dye of Lucifer yellow in human corneal cells for the estimation of the function of GJIC using Scrape loading dye transfer assay.

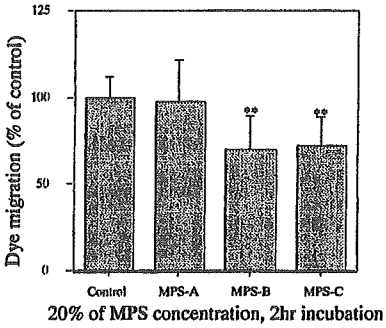

Fig.2 Determination of dye migration after scrape loading dye transfer assay using human corneal cell incubated with 20% of MPS solutions for 2 hours.

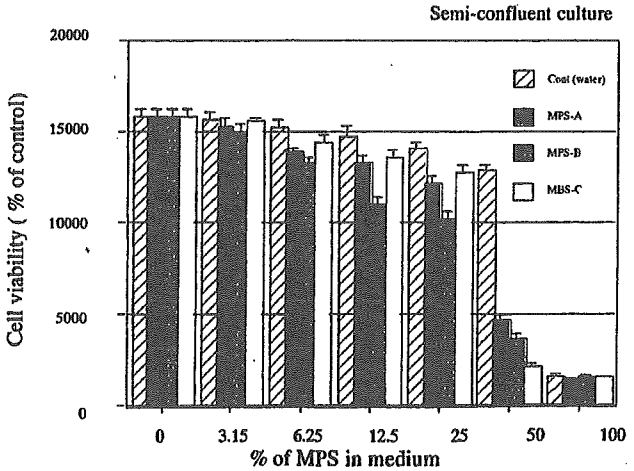

Fig.3 Cell viability of human corneal cells after exposure with various concentrations of MPS solutions using alamarBlue assay.

CHANGE OF THE CELLULAR FUNCTION BY CONNEXIN GENE TRANSFECTION IN A HEPATOMA CELL LINE

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Abstract

Connexin 32 (Cx32) is the main gap junction protein in hepatocytes and plays an important role in the regulation of liver gap junctional communication (GJIC). In this study, the human Cx32 gene was transfected in a hepatoma cell line (HepG2) that is aberrant expression of Cx32 and deficient in GJIC. Cx32-transfected HepG2 showed the increased GJIC comparing with HepG2 and the vector-transfected HepG2. Furthermore, the liver functions of ammonia removal activity of HepG2 were remarkably enhanced with Cx32 gene transfection. It may be expected to improve the cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an efficacious bioartificial liver.

Introduction

A cell-based biohybrid artificial liver (BAL) is a promising approach to support patients with acute liver failure[1]. To overcome worldwide shortage of donor organs and avoid xenozoonosis risk, a hepatoma cell line HepG2 derived form the human-origin cell has growth characteristic and are less severe antigenecity, and then has already been used for developing the BAL[2]. Although HepG2 keeps liver-specific functions well among hepatoma cell lines, the activities of the liver-specific functions in HepG2 were far lower comparing with these of primary hepatocytes[3]. On the other hand, gap junction intercellular communication (GJIC) is considered to play an essential role in the control of proliferation, differentiation and homeostasis of various cells. In the liver, hepatocytes are coupled to each others by gap junctions and GJIC is necessary for liver homeostasis growth control and signal transfer, especially related to glycogen

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