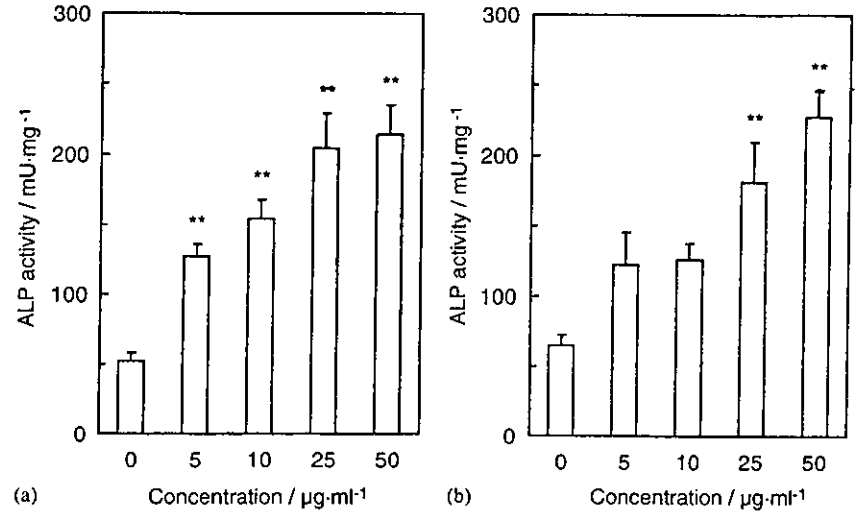

Fig. 4. The ALP activity of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The ALP activity of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using *p*-nitrophenylphosphate 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.

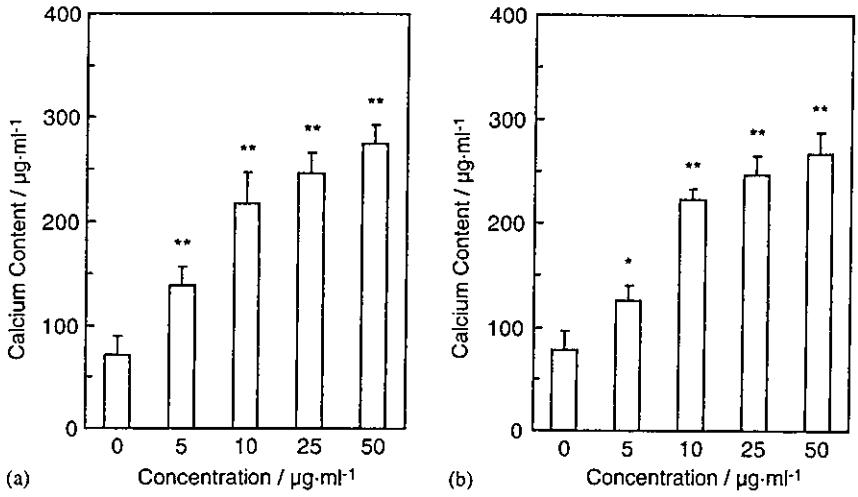

Fig. 5. The calcium content of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcium content of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using 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 immature 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.

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Neural differentiation of midbrain cells on various protein-immobilized polyethylene films

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Abstract: The effect of surface modification of polyethylene (PE) film on differentiation of midbrain (MB) cells obtained from rat embryos was determined by their micromass culture system. When cultured on untreated PE film, cell differentiation was suppressed to approximately two-thirds of that observed in a control culture dish. On the contrary, type I collagen-immobilized PE film increased differentiated foci of the MB cells more than did the untreated PE film. RGDS (Arg-Gly-Asp-Ser) peptide immobilization onto PE film resulted in almost the same differentiation activity as the collagen immobilized PE film. Bovine serum albumin (BSA) immobilization onto PE film enhance the differentiation activity more than did the untreated PE film, but not up to the levels of collagen- and RGDS-immobilized PE. The number of differentiated foci of the MB cells on untreated PE film were increased by the addition of the condition medium prepared from the collagen-immobilized PE film. However, the number of foci was not increased by the addition of other condition media obtained from control dish, untreated, BSA-, and RGDS-immobilized PE. On the other hand, none of these condition media enhanced a differentiation of the neuronal cell line of PC12 cells, suggesting that some factors effectively differentiate midbrain cells, composed of neuronal epithelial and mesenchymal cells, but not the PC12 cells secreted in the condition media prepared from collagen-immobilized PE. In addition, it is probable that neural growth factor was not secreted in these condition media, which could not induce the differentiation of PC12 cells. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 439–446, 2003

Key words: midbrain cells; cell differentiation; micromass culture system; surface modification; collagen

INTRODUCTION

Implantation of biomaterials still induces many side effects, although recent advances in technology have made it possible to improve biomaterials more safely and effectively. Undesirable inflammatory reactions were often observed around the biomaterials after their implantation, resulting in not only the loss of their expected function but also severe side effects for the patients. For studies on interactions between the biomaterials and the body, in other words, many types of cells should be carefully investigated *in vitro*. The effect of the materials on cell proliferation, differentiation, and function must be estimated in order to improve biomaterials that can be applied to humans. Previously, we reported that when cells were cultured on a polyethylene film, gap-junctional intercellular communication (GJIC) of cells, which is an important function to maintain cell and tissue homeostasis, was inhibited. Furthermore, the inhibitory level decreased when the surface of the film was modified with collagen.[1] Thus, we suggested that surface modification of biomaterial is one way to diminish undesirable effects on cell and tissue homeostasis. However, it is not clear whether cell differentiation function can be improved with recovery of the GJIC by a surface characteristic of the biomaterial. It is important to design a surface characteristic suitable for normal cell differentiation in order to develop biomaterials, such as a biocompatible scaffold, for tissue engineering. To clarify the effect of biomaterial surface characteristics on cell differentiation and the relationship of biomaterial effects on differentiation and GJIC, we applied a midbrain (MB) micromass culture to estimate the effect. The MB micromass culture system is a convenient *in vitro* assay originally developed for *in vitro* teratogenicity tests.[2] In addition, it has already been suggested that the differentiation level of the MB cells might be related to GJIC inhibitory activity of the biomaterial.[3] In this study, we examined the effect of surfaces modified with various kinds of proteins on cell differentiation under *in vitro* experimental conditions to clarify suitable surface characteristics for producing excellent biomaterials.

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MATERIALS AND METHODS

Chemicals

A high-density polyethylene (PE) film with no additives was kindly supplied by Mitsui Chemical Industries, Ltd. (Tokyo, Japan). Its thickness was 0.35 mm and the weight-average molecular weight was 153,000. We used acrylic acid monomer obtained from Nakalai Tesque Inc. (Kyoto, Japan) after conventional distillation. Bovine serum albumin (BSA) and Type-I collagen from porcine tendon (cell-matrix, type I-P) were purchased from Sigma Chemical Co. (St. Louis, MO) and Nitta Gelatin Co., Ltd. (Osaka, Japan), respectively. Arg-Gly-Asp-Ser (RGDS) peptide was kindly donated by Yoshiaki Hirano (Osaka Institute of Technology, Japan). Hanks's balanced salt solution (HBSS) and Ham's F12 medium were purchased from Life Technologies, Inc. (Grand Island, NY). We purchased and used without further purification 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (WSC) and other chemicals from Wako Pure Chemical Industries (Osaka, Japan).

Immobilization of proteins onto PE film

Protein immobilization onto the surface of PE film was carried out according to the method reported previously.[1] Briefly, we subjected PE film to corona discharge to introduce peroxides onto the surface and then placed it in a solution containing acrylic acid monomer in a Pyrex tube, followed by degassing procedure. The tube was kept at 60°C for 1 h to allow the graft polymerization of the monomer to proceed onto the PE surface. After placing grafted film in 0.1 *M* acetic acid solution (pH 4.5), we added WSC to the solution. The film was transferred to a protein solution to immobilize the protein onto the grafted film. The amount of protein immobilized onto the film was estimated by the ninhydrin method after hydrolysis of immobilized protein. The immobilized films were sterilized by immersion in 70% ethanol solution for 12 h at 4°C, and subsequently placed in sterile phosphate-buffered saline solution (PBS, pH 7.4) to remove ethanol just before the assay described below.

Cell differentiation assay by micromass culture of midbrain cells

MB micromass culture was performed according to the method previously reported.[2] Figure 1 shows the scheme of the assay of the MB micromass culture system. Briefly, MB tissues were separated from the embryos of pregnant Wister rats (Japan SLC Inc., Shizuoka, Japan) on day 13 of gestation. MB tissues were dissociated into individual cells by successive washing in calcium- and magnesium-free HBSS, and by trypsin digestion for 10 min at 37°C. The cells were suspended at a density of 5×10^6 cells per mL in culture medium consisting of Ham's F12 with 10% fetal calf serum (F12–10% FCS). Aliquots of 20 μL of cell suspension were seeded on PE film or protein-immobilized PE film placed into 12-well cell culture plates, followed by the addition of 1 mL of F12–10% FCS after 2 h and an incubation at 37°C in a 5% CO_2–95% air atmosphere for 1 wk. After the cells were fixed and stained with hematoxylin, the extent of nerve cell differentiation was assessed by counting the differentiated foci under a dissecting microscope. The differentiated cells on each test film were photographed with a digital microscope VH-8000 (Keyence Co., Ltd., Osaka, Japan). Cytotoxicity of the films was estimated by the alamar Blue® assay (BioSource International, Inc., Camarillo, CA), which incorporates an oxidation-reduction indicator based on detection of metabolic activity.[4] After 1 wk of incubation, aliquots of 50 μL alamar Blue solution were added to each test dish, followed by a further 4-hr incubation. The fluorescence intensity (excitation 530 nm, emission 570 nm) of supernatant was estimated by CytoFluor® II (PerSeptive Biosystems,

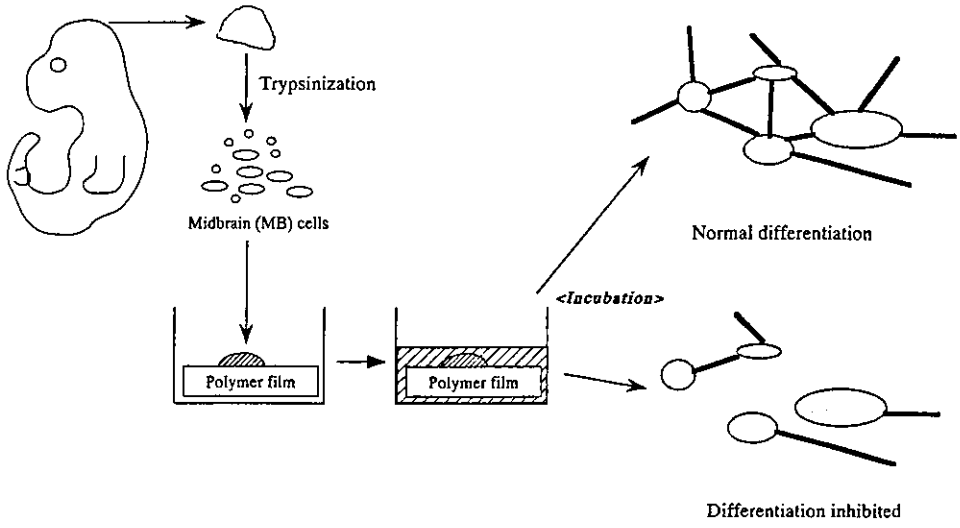

Figure 1. Schematic illustration of micromass culture system using MB cells.

Flamingham, MA). The effects of condition media of MB tissues cultured on Various PE films were estimated as follows: MB cells were collected and cultured on PE films as above, followed by incubation at 37°C in a 5% CO_2–95% air atmosphere. Then, supernatants of MB cell culture (condition media) were collected after 3 days, followed by the addition of 1 mL F12–10% FCS and an additional 4-day incubation. The condition media were collected again after cultivation of MB cells. All condition media collected were kept at –80°C until we detected an effect on differentiation and proliferation of MB cells. To detect the effect of condition medium, MB cells were subjected to the micromass culture on a normal culture dish, followed by the addition of 500 μL F12–10% FCS and 500 μL the condition medium. The effect was estimated as described above after 1-wk incubation. The experiments were repeated at least three times.

Effects of condition medium from MB cell culture on PC12 differentiation

To examine effects of the condition medium on neuronal cell differentiation, we cultured PC12 cells as model neuronal cells, using the condition mediums from MB cultured on various kinds of PE films. PC12 cells were kindly donated by Shuichi Koizumi (National Institute of Health Sciences, Tokyo, Japan) and cultured in a medium consisting of Dulbecco's modified Eagle's medium (MEM) supplemented with 5% FCS and 5% heat-inactivated horse serum. The cells were suspended in the medium at the density of 2×10^4 per mL. An aliquot of 500 μL of the suspension was added to 24-well collagen-coated culture plates, followed by a 5-h incubation to adhere the cells onto the plates. Then, an aliquot of 100 μL of the condition medium was added to each plate, and axonal growth from the cells was observed daily by light microscopy to estimate a differentiation level of PC12. For positive control of differentiation of the cell, 25 μg of nerve growth factor (NGF; purchased from Wako Pure Chemical Ltd., Osaka, Japan) was added to the plates and treated as above.

Statistic analysis

All data were expressed as the mean value ± standard deviation of the data obtained from each experiment and treated statistically with Student's *t* test.

RESULTS

Figure 2 shows foci of MB cells cultured on various protein-immobilized PE films after a 7-day incubation. MB cells formed many foci, and many neuron-like fibers were observed between the foci when cultured on polystyrene and collagen-coated culture dishes. The MB cells on untreated PE films developed few neuron fibers compared to those on the polystyrene culture dish, and the cells did not gather to create cell foci. However, a protein immobilization onto PE caused neuron fibers to connect between foci, although the number of fibers observed was affected by the kind of protein immobilized. In Figure 2, it is clear that the size of the focus was also affected by the protein immobilized. When collagen was immobilized, the observed foci were smaller than the other foci. However, the number of fibers from each focus was greater than that of the other PE film, indicating differentiation of MB cells promoted by collagen. Table I indicates the number of differentiated foci of MB cells on various PE films. The PE film inhibited differentiation of MB cells, as seen in the small number of differentiated foci. In addition, the PE film showed high cytotoxicity compared with a control dish. This may be one reason why the differentiation of MB cells was inhibited. By protein immobilization, however, the number of differentiated foci increased in comparison with those of the PE film. Moreover, collagen and RGDS peptide immobilization increased the number of the foci more than that observed on the culture dish.

We estimated the effects of condition media on MB cell differentiation to see whether they include some factors affecting cell differentiation. Table II shows the effects of condition media on MB cell differentiation on a collagen-coated culture dish. Addition of the condition medium from MB cell cultivation on all kinds of PE films showed a cytotoxic effect on MB cells. In addition, the cytotoxic effect was enhanced when collected after 7 days of incubation. This effect might be ascribed to a loss of nutrition from the medium during the first cell culture. The differentiation of MB cells on a collagen-coated dish was suppressed by the addition of the media from MB cells on various PE films on day 3. However, a condition medium from MB cell on collagen-immobilized PE film on day 7 showed many differentiated foci, as many as were induced by a condition medium from MB cells on a collagen-coated culture dish on day 7. Although the medium from the collagen-immobilized film revealed an enhanced effect on differentiation, addition of the condition media from other films suppressed differentiation, as shown in Table II. On the contrary, all condition mediums after 3-day cultivation did not show any enhancement of MB cell differentiation on a culture dish.

PC12 cells cultured with the condition medium from MB cell cultures showed little axonal growth after 1-wk incubation, and the axonal growth was similar among the various condition media of MB cells cultured on the different PE surfaces. On the other hand, the cells cultured with NGF showed strong axonal growth (data not shown), indicating that the con-

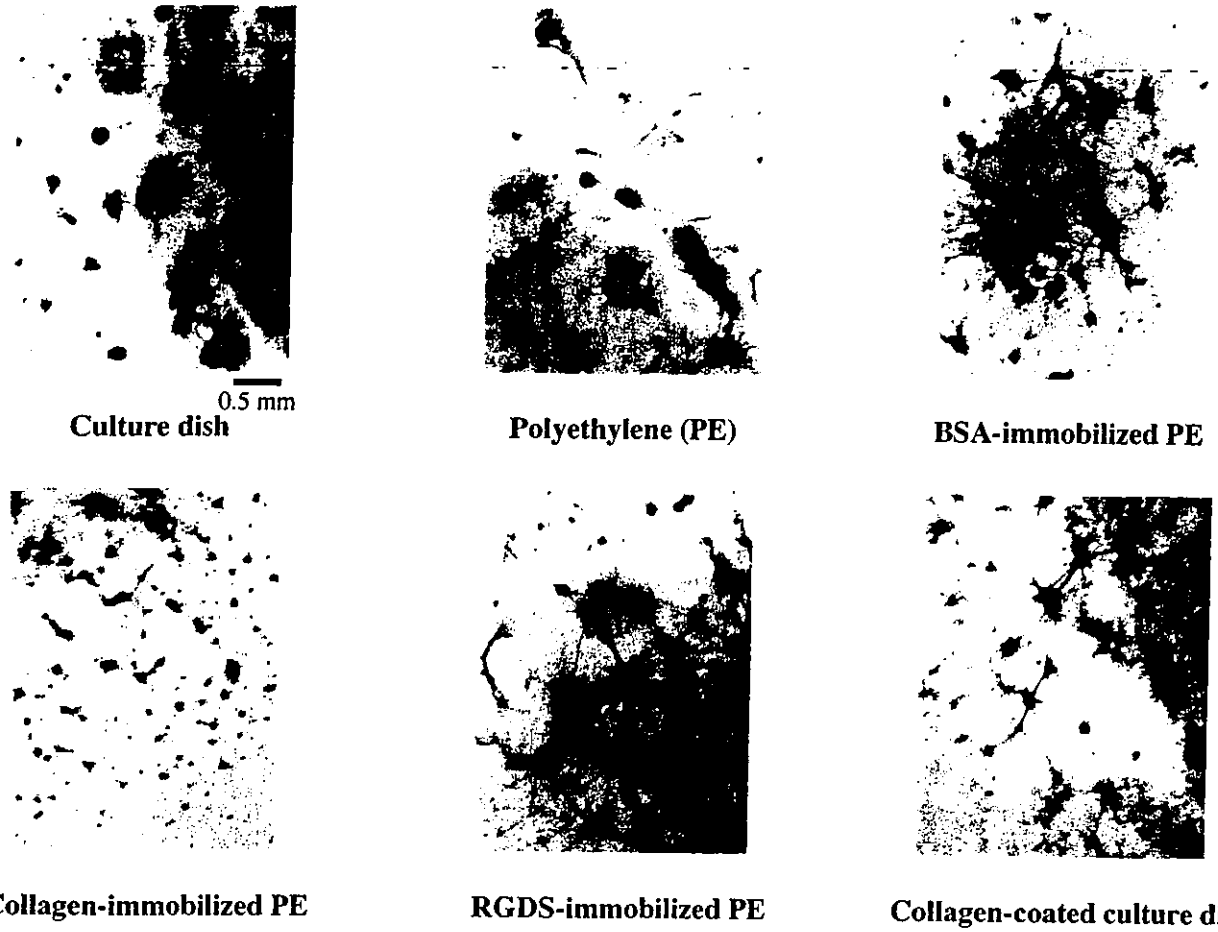

Figure 2. Light micrographs of Delafield's hematoxylin staining cultures of rat MB cells after incubation on various polyethylene surfaces.

dition medium does not contain a factor such as NGF that induces a differentiation of the PC12 cells directly.

DISCUSSION

Because function and differentiation of cells depend on circumstances such as the kinds of substrates, cell–substrate contact area, culture condition, and so on,[5–8] we should be very careful to choose the appropriate substrate for cell culture to achieve the desired function. We think this cell–substrate interaction is a very important, not only for basic cell studies but also for studies of hybrid-type artificial organs and tissue engineering. Therefore, in order to develop our tissue-engineering technique, we focused on effects of bio-

TABLE I
Effect of Surface Modification of Polyethylene Film by Protein Immobilization on Differentiation of Rat Midbrain Cells (mean value ± SD; $n = 4$)

	Culture Dish	Virgin PE	BSA-PE	Collagen-PE	RGDS-PE	Collagen-Coated Culture Dish
The amount of protein immobilized ($\mu g/cm^2$)	—	—	2.27	4.19	2.46	—[a]
Cytotoxicity (%)	100 ± 15.5	61.5 ± 9.0	75.5 ± 13.8	73.9 ± 38.3	43.4 ± 41.0	137 ± 10.6
The number of differentiated foci of MB cells	94.0 ± 16.6	67.0 ± 17.4	109 ± 26.5	159 ± 16.1**	168 ± 22.7**	174 ± 15.4[b]

[a]The coated amounts of collagen unknown.
[b]Significant difference observed among culture dish, PE, and BSA-PE.
*$p < 0.05$, **$p < 0.01$

TABLE II
Effect of Condition Media From Various MB Cell Culture Conditions on MB Cell Differentiation on a Collagen-Coated Culture Dish (mean value ± S.D. $n = 4$)

	Condition Medium Collected After	Condition Medium Collected From MB Culture				
		Collagen-Coated Culture Dish	PE	BSA-PE	Collagen-PE	RGDS-PE
Cytotoxicity (%)	3 days	100 ± 6.8	88.7 ± 9.4*	75.5 ± 13.8*	68.4 ± 9.9*	77.1 ± 15.2*
	7 days	100 ± 28.1	64.3 ± 15.9	39.1 ± 17.2**	50.5 ± 10.3*	42.7 ± 28.0**
Number of differentiated foci of MB cells	3 days	126 ± 12.3	109 ± 23.0	107 ± 6.4	100 ± 5.5	110 ± 10.4
	7 days	133 ± 9.3	105 ± 14.2*	90.0 ± 25.5*	133 ± 13.0	87.0 ± 61.8*

*$p < 0.05$, **$p < 0.01$ compared with a collagen-coated culture dish group or with group indicated.

materials on cell differentiation to estimate biocompatibility of materials. Moreover, we expected to find suitable cell scaffolds or substrates for inducing desirable function of targeted cells. In this study, PE film inhibited normal differentiation of MB cells cultured on it, and collagen and RGDS peptide immobilization onto PE surface resulted in an increased number of differentiated foci. In addition, MB cells on a collagen-coated culture plate showed more differentiated foci than those on a normal culture dish, as well as those observed on collagen-immobilized PE film. These results suggest that improvement of cell adhesion and cell proliferation by the immobilization of extracellular matrix protein promotes the differentiation of MB cells.

It has been reported that some materials show a potential to disrupt usual behavior of cells, resulting in undesirable effects on cells or tissues.[1,2,9–12] During these studies, we focused on the inhibitory activity of the materials on GJIC, which was found to play an important role in tumor promotion activity and correlated well with *in vivo* tumor potential.[10,11] In addition, GJIC plays an essential role in homeostasis maintenance by keeping many growth control signals at equilibrium among GJIC-connected cells and tissues.[13] Therefore, it is important to clarify the inhibitory activity of the materials on GJIC to estimate both their biocompatibility and their undesirable effects *in vivo*. In our previous study, PE film showed strong inhibitory activity on GJIC in metabolic cooperation assay systems. This inhibitory activity decreased when a surface of PE film was immobilized with extracellular matrix proteins such as collagen. However, surface modification of PE by immobilization of RGDS peptide, which plays an important role in cell adherence to the protein via integrin molecules on cell membrane, did not improve its inhibitory activity.[14] This result suggested that RGDS sequence in the protein was not sufficient to recover normal cell homeostasis via GJIC. Therefore, we expected that PE film immobilized with RGDS peptide would suppress normal cell differentiation of MB cells when cultured on the film, as well as on untreated PE film. This study showed that untreated PE film inhibited cell differentiation as expected. However, RGDS-immobilized PE showed a potential to differentiate the cells more than that on a control dish. There were many kinds of cells in this MB cell culture system, because primary cells collected from mouse embryos have been suggested to differentiate nerve cells on the layer of other cells, such as mesenchymal cells, in this system.[2] It is possible that growth of mesenchymal cells was improved by the surface modification with the cell-adhesive RGDS peptide, resulting in improved differentiation of the nerve cells. This suggests that interaction between mesenchymal cells normally grown on a substrate, and nerve cells on the layer of the mesenchymal cells, is one of the important factors in the differentiation of MB cells.

Through microscopic observations (Fig. 2), we observed that the number and size of MB cell foci on various kinds of PE films were different. For example, MB cell size was smallest when the cells were cultured on collagen-immobilized PE. On the other hand, when the MB cells were cultured on untreated PE film, the number of foci was fewer and, as observed, their size was larger than the foci seen on other films. In addition, many cell layers uniformly stained by hematoxylin were observed on the PE film. These findings indicate that MB cells could not gather to form foci when cultured on the PE film. However, improving the surface of the PE by protein immobilization recovered mobility of the cells to form the foci, judging from the microscopic observation. These results suggest that protein immobilization makes the surface more feasible for both cell movement and adherence. Considering this microscopic observation and data shown in Table II, it is probable that more nutrition is necessary for adhesion, movement, proliferation, and differentiation of MB cells on various kinds of PE films tested

rather than on a normal culture dish. Although further studies are needed, differences in surface characteristics of various PE films may cause different biological reactions in MB cells, resulting in different utilization of the nutrition in a culture medium.

Despite containing less nutrition, the condition medium from MB cell 7-day cultures on collagen-immobilized PE film might have the potential to improve differentiation of nerve cells, as shown in Table II. This suggests that the condition medium contains some factors that aid differentiation. Results from Table II also suggest that growth factors from the cells that aid differentiation may be secreted at least 3 days after starting MB cell culture, because the condition medium from 3-day cultures did not show any effects on differentiation. However, culture of PC12 cells in the condition medium indicated that growth factors in the medium had little NGF-like potential on differentiation. These results suggest the possibility of improving function of mesenchymal or nerve cells on the collagen-immobilized PE film to produce not NGF, but factors that may induce normal nerve cell differentiation, although the factors were not identified in this study. In addition, the condition medium from the 7-day cultures on other protein-immobilized films suppressed differentiation, although the number of differentiated foci directly on the films was more than that observed on PE film. This finding suggests that the condition medium did not contain the factors. In addition, this may also reflect lower nutrition in the condition medium than that taken from MB cell cultures on a normal culture dish. These results indicate that mechanisms of MB cell differentiation recovery on collagen-immobilized PE film are different from those of RGDS-immobilized PE. The hypothesis of MB cell differentiation on various PE films from the above findings is illustrated in Figure 3. Immobilization of protein makes a surface more hydrophilic. It is known that a hydrophobic or hydrophilic surface of biomaterials is one factor that affects cell adhesion behavior and function on them.[15] This change in surface characteristic can improve MB cell differentiation through normal adhesion and rearrangement of mesenchymal cells on PE surface. Because collagen and RGDS sequences can bind to a cell through integrins in the cell membrane, it is probable that the rearrangement of mesenchymal cells induces collagen- or RGDS-immobilized PE rather than BSA-immobilized PE. Moreover, collagen-immobilized PE, on which cells are reported to recover GJIC function, induces production of unknown factors that improve MB cell differentiation. Although we did not estimate directly the GJIC function of MB cells on various PE films, it is likely that the inhibitory activity of the PE films on the

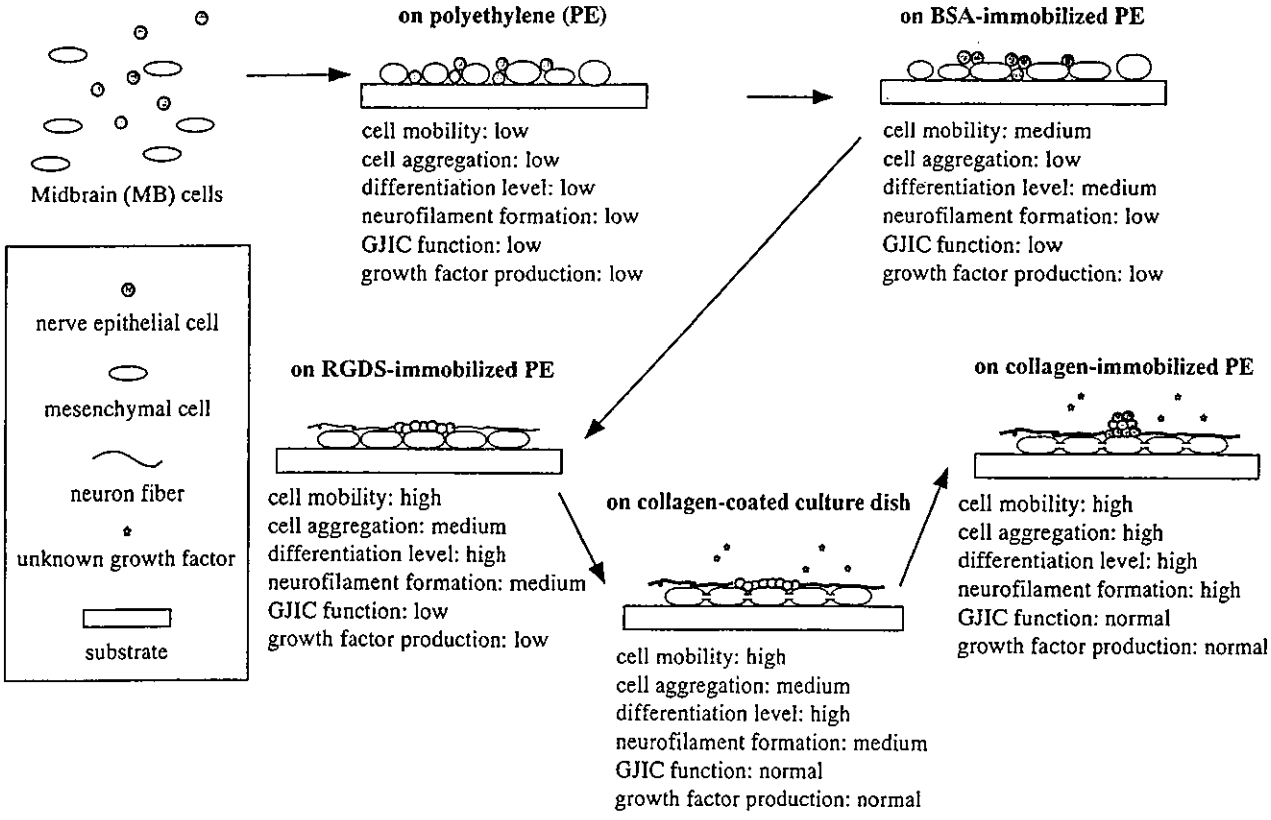

Figure 3. Schematic illustration of assumed differentiation mechanisms of MB nerve cells and mesenchymal cells after cultivation on various kinds of PE films.

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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A novel function of connexin 32: marked enhancement of liver function 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 signal transfer and growth control in the liver by constructing gap junction channels and gap junctional intercellular communication (GJIC). In this study, the human Cx32 gene was transfected into a hepatoma cell line (HepG2) that showed aberrant expression of Cx32 and was deficient in GJIC. Cx32-transfected HepG2 not only expressed a higher level of Cx32 mRNA, but also showed increased GJIC compared with HepG2 and vector-transfected HepG2. Furthermore, the liver functions of ammonia removal and albumin secretion of HepG2 were markedly 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.

Keywords: Connexin; GJIC; Liver functions; Hepatoma cell line; HepG2

Gap junctions are transmembrane channels linking neighboring cells and providING the only pathway to transfer small hydrophilic cytoplasmic metabolites less than 1000 Da, growth modulators, and second messengers between the adjacent cells, in a process known as gap junctional intercellular communication (GJIC) [1]. GJIC was suggested to play a crucial role in maintaining tissue homeostasis and controlling growth, differentiation, embryogenesis, and several functions of different tissues [2–4]. Gap junctions are composed of two hemichannels and each hemichannel consists of six connexin (Cx) protein units. At present, there are greater than 16 different Cxs in vertebrate species and expression of some Cxs is organ specific [5]. In the liver, GJIC involves at least three different connexins, Cx32, Cx26, and Cx43, depending on the cell type and cell position in the lobule [6]. In vivo, Cx32 and Cx26 are expressed in parenchymal hepatocytes and the distribution of these Cx proteins is different within the liver lobules: Cx26 preferentially localizes in the periportal zone of the lobules, whereas Cx32 appears in most hepatocytes throughout the lobules and is the major component of liver gap junctions. Furthermore, many biological activities of the liver are spatially organized within the circulatory unit and several hepatic functions differ in periportal vs. pericentral hepatocytes, including carbohydrate, lipid, and nitrogen metabolism in addition to expression of gap junctions. Recently, several studies suggested that Cx32 expression had an inhibitory effect on hepatocarcinogenesis and transfection with Cx32 cDNA inhibits the growth of hepatoma cells [7–9]. However, it was not clear whether the recovery of GJIC by transfection of Cx32 gene would enhance the liver-specific functions of hepatoma cells, which would be very important in the research of liver disease therapy.

In the last two decades, with the development of cell biology and tissue engineering, a cell-based biohybrid artificial liver (BAL) was reported to be a promising approach to support patients with acute liver failure [10]. Primary human hepatocytes would be ideal for the cellular component of BAL, but it was limited by the

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worldwide lack of donor organs and the growth limitations of primary hepatocytes in vitro. To overcome the shortage of donor and avoid xenozoonosis risk, a hepatoma cell line (HepG2) derived from human-origin cells has good growth characteristics and less severe antigenicity, and was previously used for developing the BAL [11]. Although HepG2 maintains good liver-specific functions among hepatoma cell lines, the activities of the liver-specific functions of HepG2 were far lower compared with those of primary hepatocytes [12]. Cx32 is the major gap junction protein expressed in hepatocytes, but HepG2 is an aberrant expression of Cx32 and is deficient in GJIC. Therefore, we transfected the Cx32 gene into HepG2 and investigated the exchanges of GJIC and liver-specific functions of HepG2 in this study.

The results showed that Cx32 gene transfected in HepG2 improved the trafficking of Cx32 protein to the cytoplasmic membrane, clearly increased the GJIC, and enhanced the activities of ammonia removal and albumin secretion in the Cx32 gene transfected HepG2. This was the first finding that Cx32 could markedly enhance the liver-specific functions in a hepatoma cell line (HepG2).

Materials and methods

Cell culture. The human hepatoma cell line HepG2 from the Riken cell bank (Tokyo, Japan) was cultured at 37 °C under 5% CO_2/95% humidified air using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM non-essential amino acids (NEAA) (Gibco), 10% fetal bovine serum (FBS) (Intergen, NY), and 100 U/ml penicillin–streptomycin (Gibco).

Plasmid construction and transfection. Using genomic DNA extracted from HepG2 as template, the human connexin genes were amplified by polymerase chain reaction (PCR) using primers Cx32F (5′-ATGAACTGGACAGGTTTGTAGACCTTGCTC-3′) and Cx32R (5′-TCAGCAGGCCGAGCAGCGG-3′). These amplified gene fragments were isolated and inserted into the pTARGET mammalian expression. HepG2 cells were transfected with the Cx32/pTARGET plasmid or empty vector as a control using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions with minor modification. After continuously culturing for two days, transfectants were selected by adding 1.3 mg/ml geneticin (Life Technologies, Frederick, MD) in the culture medium for one week. Individual transfected clones were prepared by limiting dilution cloning in 96-well plates and then culturing as for HepG2.

RT-PCR. Total RNA was isolated from cells cultured on the seventh day with TRIzol reagent according to manufacturer's instructions. The cDNA was prepared from 1 μg of total RNA by reverse transcription using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After proper optimization of PCR conditions, subsequent PCR was performed with 1 μl cDNA in 20 μl reaction mixture (10× PCR buffer 2 μl, dNTP 1.6 μl, each primer 2 μl, *Taq* DNA polymerase 0.2 μl, and distilled water). The conditions for RT-PCR were equilibration at 37 °C for 15 min, followed by an initial denaturation at 95 °C for 1 min, 25 cycles of 95 °C for 1 min, 60 °C for 1 min, 70 °C for 2 min, and final extension of 70 °C for 5 min. Electrophoresis of PCR products was done on 1.5% agarose gel for the visualization of connexin after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). Images were captured using an image scanner and analyzed using NIH Image software. The primers used in this study were as follows:

hCx32	forward 5′-ATGAACTGGACAGGTTTGTACACCTTGCTC-3′
	reverse 5′-TCAGCAGGCCGAGCAGCGG-3′
hCx26	forward 5′-ATGGATTGGGGCACGC-3′
	reverse 5′-TTAAACTGGCTTTTTTGACTTCCC-3′

Immunocytochemical stainings. Immunocytochemical staining of Cx32 protein was performed using the VECTASTAIN ABC kit (Vector Laboratories, Inc. Burlingame, USA) following the manufacturer's instruction with some modification. Briefly, cells grown on the glass coverslips were fixed in cold pure acetone for 5 min. The acetone-fixed specimens were blocked in diluted normal blocking serum in Dulbecco's phosphate-buffered saline (PBS) at room temperature for 30 min and incubated with polyclonal rabbit anti-connexin 32 (Zymed Laboratories, San Francisco, CA) overnight at 4 °C. Protein–antibody complexes were visualized by the biotin/streptavidin/peroxidase method with diaminobenzidine tetrahydrochloride (DAB) (Vextor Laboratories, Burlingame, USA) as the chromogen. All slides were viewed with a Nikon microscope (Nikon, Japan).

Scrape-loading/dye transfer assay to measure GJIC. The scrape-loading/dye transfer (SLDT) technique was adapted after the method of El-Fouly et al. [13]. Briefly, when the cells grew into confluent monolayer cells in 35-cm dishes, cell dishes were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA) in PBS (+) solution and scraped immediately with a sharp blade after rinsing with PBS (+). After incubating for 5 min at 37 °C, cells were washed with PBS (+) and monitored using a fluorescence microscope. The dye spreading distance was measured from the cell layer at the scrape to the edge of the dye front that was visually detectable.

Liver-specific function assay. The functions of the hepG2 and Cx32 transfected cells were evaluated by measuring ammonia removal and albumin secretion. For the ammonia removal activities of these cells, the cells were cultured in MEM with 5 mM ammonium chloride. After the exchange of the medium containing ammonium, the concentration of ammonia in the medium was measured at 0 and 24 h, respectively, using the indophenol method (an ammonia assay kit, Wako Pure Chemicals, Japan). The albumin secreted into the culture medium was detected by enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA).

Statistical analysis. Student's *t* test was used to compare the samples. Statistical significance was represented by $p < 0.05$. Values were means ± SD. Three cultures were run for each case and all experiments were repeated at least twice.

Results

Functional GJIC in HepG2 enhanced by Cx32 gene transfection

HepG2 cells were transfected with Cx32/pTARGET plasmid DNA using FuGENE6 transfection reagent and the transfectants were obtained by selection with geneticin. Expressions of Cx mRNAs were first detected using RT-PCR (Fig. 1). As shown in Fig. 1A, Cx32 mRNA was detected and showed different levels among the HepG2, Cx32 gene- and empty vector-transfected cells, while the Cx26 mRNAs were almost not detected in all cells. The image analysis showed the level of Cx32

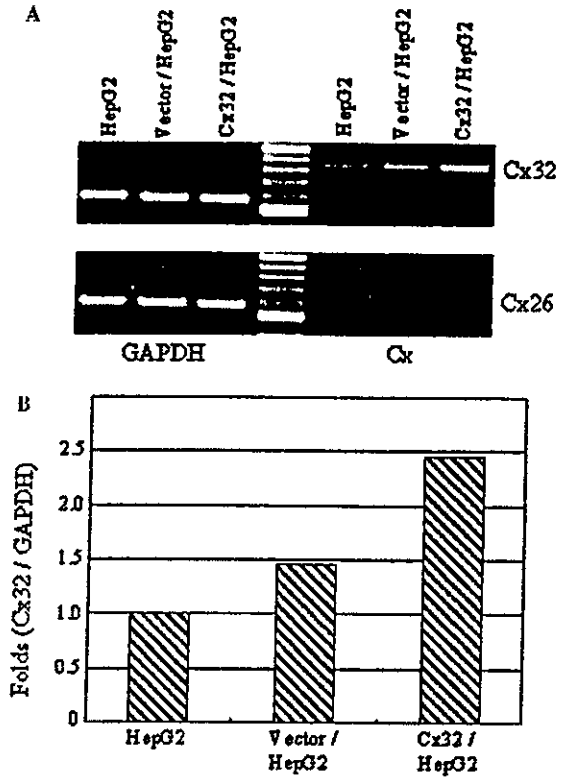

Fig. 1. Establishment of Cx32 and vector transfected HepG2. (A) RT-PCR analysis of Cx32 and Cx26 gene expression in HepG2, vector-transfected cells (Vector/HepG2), and Cx32 gene transfected cells (Cx32/HepG2). (B) Image assay of Cx32 gene expression in RT-PCR. Relative densities were standardized to that of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

mRNA expressed in the Cx32 gene transfected cells was enhanced 2.5- and 1.7-fold in comparison with the HepG2 and empty vector transfected cells, respectively (Fig. 1B). The abilities of functional GJIC in the cells were investigated by the scrape-loading dye transfer technique. The distances of lucifer yellow spreading reflected the functional GJIC in the cells, and the longer distance of dye spreading indicates the higher functional GJIC in the cells. As shown in Fig. 2, the distance of lucifer yellow spreading in Cx32 gene transfected cells was clearly greater than those in HepG2 and empty vector transfected cells. Thus, the distance of dye spreading in Cx32 gene transfected cells was 2.8- and 1.8-fold longer than those in HepG2 and empty vector transfected cells, respectively (Fig. 3). It could be concluded that the Cx32 gene transfection not only increased the expression of Cx32 mRNA, but also significantly enhanced the functional GJIC in HepG2.

Localization of Cx32 protein before and after Cx32 gene transfection

To confirm the contribution of Cx32 protein for the formation of functional GJIC after the Cx32 gene transfection, the localizations of Cx32 protein in the cells were further observed by immunocytochemical staining. The photographs of A, B, and C in Fig. 4 show the localization of Cx32 protein in HepG2, empty vector transfected cells, and Cx32 gene transfected cells, respectively. These results demonstrated that the Cx32 protein was expressed in all the cells, but the localizations of Cx32 protein were clearly different among them. Thus, the Cx32 protein was localized in the cell borders and formed many small gap junction plaques in the Cx32 gene transfected cells, however, the Cx32 protein was limited in the cytoplasm and hardly detected the gap junction plaques in the HepG2 and empty vector transfected cells. The results in the present study suggest that the trafficking of Cx32 protein to the cell membrane in HepG2 was enhanced by Cx32 gene transfection and then increased the functional GJIC in Cx32 gene transfected cells.

Liver-specific functions in HepG2 improved by Cx32 gene transfection

For determining the effect of Cx32 gene transfection on the liver-specific functions in HepG2, the albumin secretion ability and ammonia removal activity were continuously monitored in the HepG2, empty vector transfected cells, and Cx32 gene transfected cells, respectively (Fig. 5). Albumin secretion, which was used as a marker for protein synthesis in the liver, showed greater amounts of albumin detected in Cx32 gene transfected cells than HepG2 and empty vector transfected cells (Fig. 5A). Furthermore, ammonia removal activity, which represents the detoxification potentiality of the liver, was significantly higher in the Cx32 gene transfected cells than HepG2 and empty vector transfected cells during the 14 days of culture with 5 mM ammonium chloride (Fig. 5B). It was suggested that the small molecular ammonium was effectively eliminated through the gap junctional channels formed by Cx32 in HepG2. The enhancement of liver-specific functions of HepG2 was suggested to relate to the increasing functional GJIC by Cx32 gene transfection.

Discussion

HepG2 cells, a human hepatoma cell line, are deficient in GJIC due to the aberrant expression of Cx32 and low expression of Cx26. In in vivo and in vitro models, low or no functional GJIC was observed in various kinds of hepatocarcinoma and hepatoma, which was suggested to be involved in the malignant phenotype of cancer and tumor cells [14]. In vivo, normal rodent hepatocytes express Cx32 and Cx26, but only Cx32 expression is constant across the liver lobule [6]. Thus, liver gap junction channels composed of Cx32 are

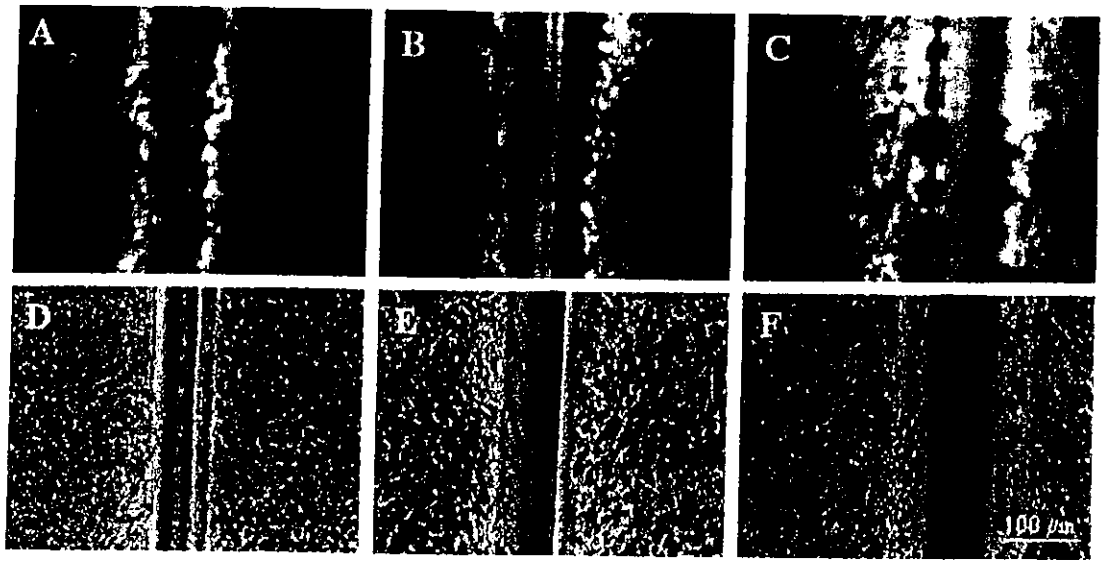

Fig. 2. Fluorescent (A–C) and phase-contrast (D-F) photographs of HepG2 (A and D), vector-transfected HepG2 (Vector/HepG2) (B and E), and Cx32-transfected HepG2 (Cx32/HepG2) (C and F) in the assay of scrape loading and dye transfer (SLDT), same scale in (A–F).

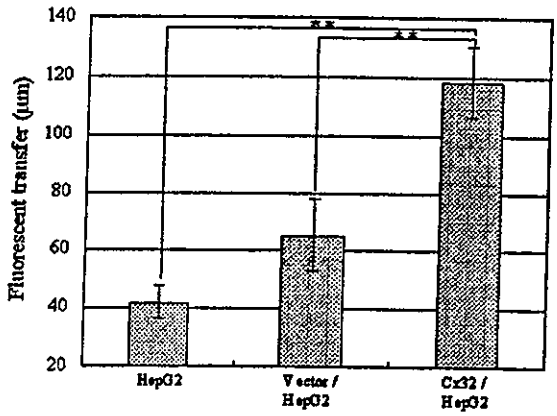

Fig. 3. Functional GJIC in HepG2, vector-transfected HepG2 (Vector/HepG2), and Cx32-transfected HepG2 (Cx32/HepG2) measured by the SLDT method at the seventh day of culture. Values are expressed as means from 20 determinations (**$P < 0.01$).

suggested to be important to maintain the normal phenotype of hepatocytes. Therefore, to reduce malignant phenotype and improve liver-specific functions in HepG2, we transfected human Cx32 gene into HepG2 to enhance functional GJIC.

The results of the RT-PCR and SLDT assay in the present study showed that the levels of Cx32 mRNA expressed in Cx32 gene transfected cells were increased greater than twofold compared with HepG2 (Fig. 1) and the functional GJIC was also markedly enhanced by Cx32 gene transfection in HepG2 (Figs. 2 and 3). Analyses of chemically induced rat liver tumors suggest that Cx32 gene is rarely mutated in these tumors but the expression of the Cx32 protein is often reduced or the Cx32 protein is abnormally localized in these cells [15]. In the present study, although the Cx32 protein expression by Western blotting assay showed almost no change even after Cx32 gene transfection (data not shown), a clear difference in the localization of Cx32 protein was observed between before and after Cx32

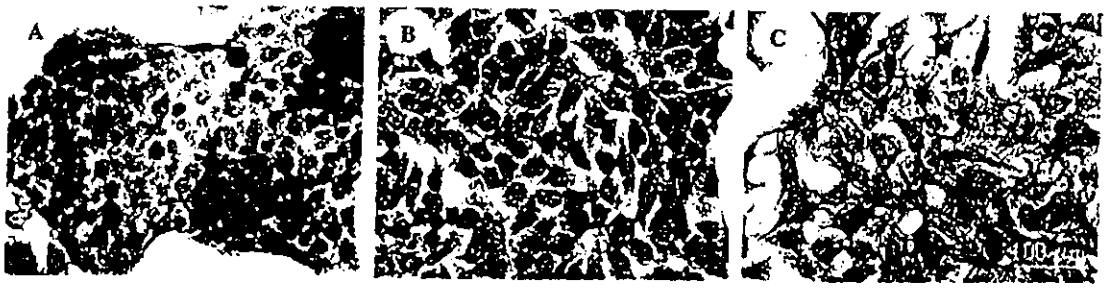

Fig. 4. Localization of Cx32 protein in HepG2 (A), vector-transfected HepG2 (B), and Cx32-transfected HepG2 (C). More gap junction plaques were detected in Cx32 gene transfected cells (arrow) than HepG2 and empty vector transfected cells. Immunocytochemical staining of Cx32 protein was performed with a Vectastain ABC Kit and polyclonal rabbit anti-connexin 32, same scale in (A–C).

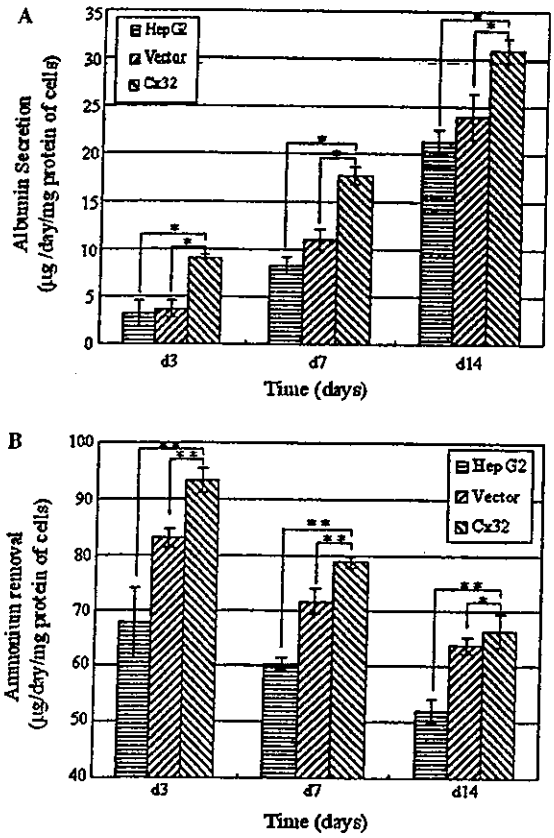

Fig. 5. Liver functions of albumin secretion (A) and ammonia removal (B) of HepG2, vector-transfected HepG2, and Cx32-transfected HepG2 examined on the 3rd, 7th, and 14th day of culture (*$P < 0.05$, **$P < 0.01$).

gene transfection in HepG2 by immunocytochemical stainings (Fig. 4). The majority of Cx32 protein was localized in the cell borders and formed small gap junction plaques in the Cx32 gene transfected cells, whereas it was limited to the cytoplasm and nucleus before Cx32 gene transfection. Furthermore, the morphologies of the cells showed that Cx32 gene transfected cells grew as a monolayer with the spreading cell shape, while the HepG2 grew as clusters with the spherical cell shape. Cx protein expression, gap junction assembly, and its function are controlled by the transcription, translation, and post-translational modification. In addition, recent studies have suggested that they are also associated with tight junction components and elements of the cytoskeleton [16–18]. Although the precise role of the cellular morphology in gap junctional channel formation between the cells is not clear at present, the results of the present study suggest that the trafficking, assembly of Cx32, and functional GJIC in the cellular membrane are enhanced by forced expression of Cx32 in HepG2.

Furthermore, Cx32 gene transfection into HepG2 markedly enhanced the liver-specific functions of ammonia removal and albumin secretion with accompanying increase in the functional GJIC (Fig. 5). Ammonia removal activity and albumin secretion ability are typical differentiated functions of the liver, but these functions in HepG2 were significantly lower than those of hepatocytes in vivo. The defect in albumin production was reported to be due to the reduction or absence of albumin gene transcription in some hepatoma cells, and the structure of the albumin gene was detected in all MH1C1, FAO, and 3924A rat hepatoma cells, but a different albumin expression was found to correlate well with methylation state of the albumin gene [19]. The results in the present study showed that the transcription of albumin gene in HepG2 may be enhanced with the increase in the functional GJIC by Cx32 gene transfection, and the albumin production was increased (Fig. 5A). In addition, the studies of ammonia removal activity in HepG2, Cx32 gene transfected cells, and vector transfected cells showed that urea was not detected in the culture media of all cells, and the ability of ammonia removal was higher in the absence than the presence of 4 mM glutamine in the media in all cells (data not shown). In the intact liver, the two major ammonia-detoxification systems, urea and glutamine synthesis, are anatomically present in periportal and pericentral hepatocytes, respectively [20]. In functional terms, this organization represents the sequence of a periportal low-affinity but high-capacity system (ureogenesis) and a pericentral high-affinity system for ammonia detoxification (glutamine synthesis). Therefore, the present results suggested that HepG2 eliminated ammonia via the high-affinity pathway of glutamine synthesis, and the capacity enhanced with the increase in the functional GJIC by forced expression of Cx32, which could be similar to the characters of Cx32 high-expressional pericentral hepatocytes. Furthermore, glutamine is an essential nutrient as a major source of energy and nitrogen for mammalian cells, which would be useful for the development of bioartificial liver. These results showed that the ammonium metabolic activity and albumin secretion in HepG2 were related to the functional gap junctional channel composed of Cx32. proteins. Other studies reported distinct biological roles of the highly homologous Cx proteins in correlations of Cx mRNA isoform expression with the degree of hepatic cellular differentiation (in RLC, FTO.2B, and WB-F344 cell lines), and suggested that Cx gene expression may be a marker of hepatic development: as hepatocytes differentiate, the proportions of Cx43 and then Cx26 mRNA decrease while that of Cx32 mRNA increases [21]. Moreover, the diffusion of second messengers through gap junction channels composed of Cx32 in liver is suggested to be a major determinant for the establishment of metabolic coupling between neighboring hepatocytes and for the proper distribution of signals involved in the promotion of liver-specific functions.

Taken altogether, the recovery of Cx32 expression could be proposed to have an effect on enhancing the liver-specific functions in hepatoma cells, in addition to improving the biological safety of hepatoma cells for the application as tissue engineered artificial liver by the inhibition of malignant growth of tumor cells of HepG2.

In conclusion, this study is the first to report a clear increase in the functional GJIC in HepG2 by transfection of Cx32 gene, and the subsequently enhanced liver-specific functions of ammonia detoxification and albumin synthesis in the Cx32 gene transfected HepG2. It may be expected to improve cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an excellent biohybrid-artificial liver.

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医療材料・医療機器の安全性と生体適合性

Safety and Bio compatibility of Bio materials and Medical devices

編集：土屋利江

Edited by Toshie Tsuchiya

シーエムシー出版

はじめに

平成17年度，大幅な薬事法の改正が施行される。これは　医療材料・機器に係わる安全対策の抜本的見直しに伴うもので，人体へのリスクに応じた安全対策を講じるための医療材料・機器のクラス分類を行うとともに，低リスク医療機器に係わる第三者認証制度や，高リスク医療機器に係わる販売許可制度が導入される。

組織工学医療用具は，細胞組織医療機器として分類され，また生物由来の医療機器・医薬品はヒトや動物由来の原料を用いた製品によるエイズやヤコブ病感染問題の反省を受けて，生物由来製品というカテゴリーを新設し，感染防止対策を柱とした改正となる。

細胞組織医療機器のみならず，新医療材料・機器も健全に発展する上で安全性・有効性評価に関わる基盤的技術の確立や，試験法・ガイドライン等の徹底は特に必要であり，企業サイドや大学研究者サイドからもそれを要望する声が大きい。

本書は，これらを踏まえた上で，安全で有効な医療材料・機器をできるだけ低コストで普及させるための第一歩として，医療材料・機器の生物学的・力学的試験法や生体適合性，安全性，品質評価技術，ガイドラインの具体的内容をまとめ，無駄な試験や誤った解釈を減らすことを目的として企画したものである。

国立医薬品食品衛生研究所および産・官・学の研究者が執筆した。本書が活用され，低価格でより安全，より有効性の高い医療材料・機器が開発され，悩んでおられる方々が一刻も早くその恩恵を享受できることが本書の願いである。

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Decreased Tumorigenicity *In Vivo* When Transforming Growth Factor β Treatment Causes Cancer Cell Senescence

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We have previously reported that transforming growth factor β (TGF-β) triggers two independent senescence programs, 1) replicative senescence dependent upon telomere shortening and 2) premature senescence independent of telomere shortening, in the cell line of A549 human lung adenocarcinoma. In this study, we examined the possibility that cancer cell tumor phenotypes could be suppressed by forced senescence. We used A549 cells treated with TGF-β for a long time (over 50 days), where senescence was induced in a telomere-shortening-dependent or an independent way. Fully senescent A549 cells were elongated, acquired contact inhibition capabilities when reaching confluence, and secreted the senescence-associated cytokine IL-6. Furthermore, senescent A549 cells had no tumorigenicity in nude mice. These results indicate that the forced induction of senescence in cancer cells may be a novel and potentially powerful method for advancing anti-cancer therapy.

Key words: cellular senescence; tumor suppression; transforming growth factor β1 (TGF-β); telomere; telomerase

Higher organisms have evolved at least two cellular mechanisms to suppress the proliferation of cells at risk for carcinogenesis: apoptosis and cellular senescence. Cellular senescence is similar to apoptosis in that cellular senescence irreversibly arrests cell growth and is a major barrier that cells must overcome in order to progress to malignancy.[1,2] However, those two cellular responses are different from each other in that apoptosis kills and eliminates potential cancer cells, while cellular senescence only arrests growth. Cellular senescence was first recognized as a biological process that prevents normal human fibroblasts from growing indefinitely in culture. This process is now known to be driven by telomere shortening and is named replicative senescence. Recently, stimuli inducing DNA damage, chromatin remodeling, and a strong mitogenic response were shown to trigger another type of programmed senescence in normal cells, which is known to be independent of telomere shortening, and is named premature senescence.[1,3–7] All these stimuli that induce cellular senescence also have the potential to cause or contribute to cancer. Thus, cellular senescence appears to be a safety mechanism for irreversibly arresting the growth of cells at risk for tumorigenesis.

Concomitant with its role in suppressing cancer, cellular senescence is found to be controlled by several tumor suppressor genes such as p53 and pRB.[8,9] Cellular senescence involves the activation of several tumor suppressor proteins and inactivation of several oncoproteins *via* the p53 or pRB pathway. Furthermore, evidence demonstrating links between cellular senescence and these tumor suppressor pathways has also been obtained by using genetically modified mice. Cells derived from mice in which the genes encoding p53 or INK4a proteins are inactivated fail to undergo senescence in response to multiple stimuli, and become cancerous at an early stage.[10] By contrast, a genetic manipulation that causes premature senescence in mammary epithelial cells suppressed the development of breast cancer in mice exposed to the mouse mammary tumor virus.[11]

We have previously clarified that transforming growth factor β1 (TGF-β) triggers two independent cellular senescence programs in cancer cells, replicative senescence and premature senescence.[12] In this study, we tried to demonstrate the link between cellular senescence and tumor suppression using the TGF-β-induced cellular senescence system.

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Abbreviations: TGF-β, transforming growth factor β1; FBS, fetal bovine serum; hTERT, human telomerase reverse transcriptase; TRF, terminal restriction fragment; SA-β-Gal, senescence-associated β-galactosidase