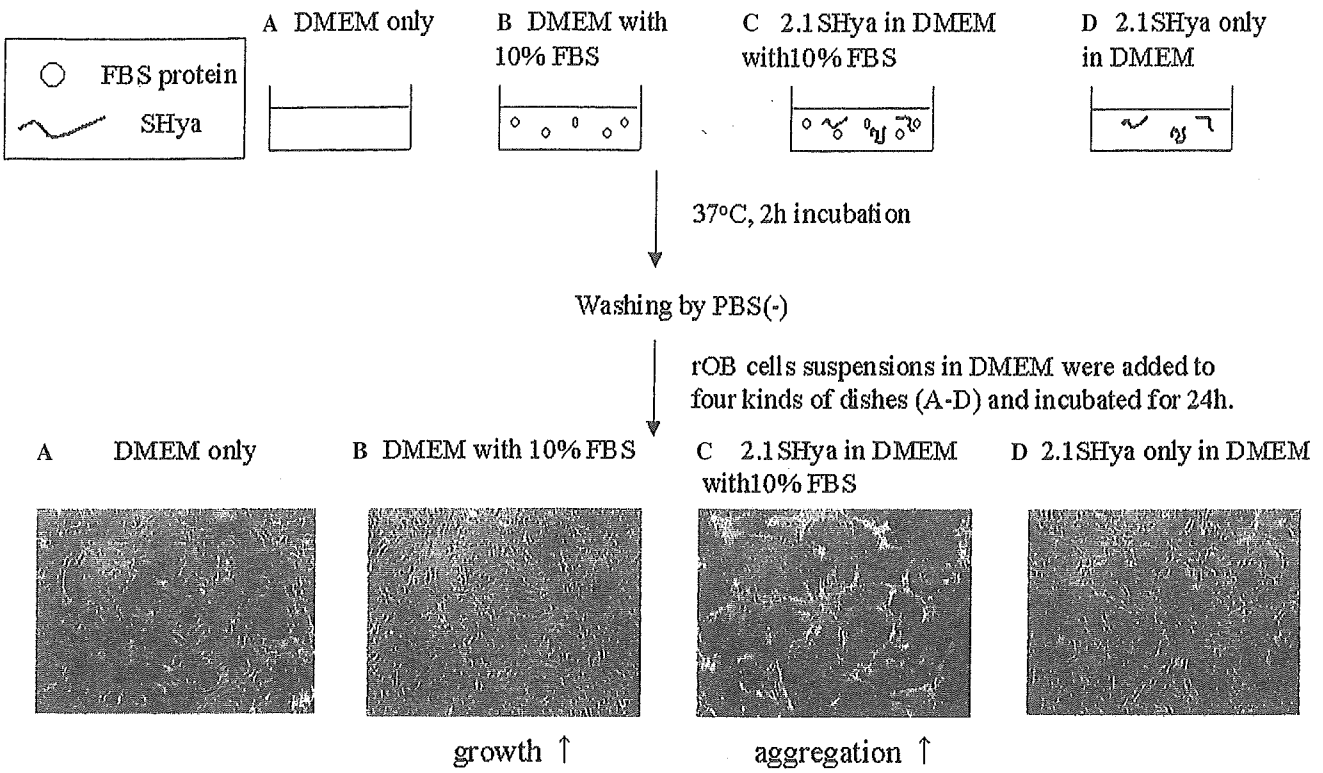

Fig. 8. Effect of FBS and 2.1SHya on cellular adhesion after 24 h. Four kinds of dishes were prepared as follows: (A) DMEM only, (B) DMEM with 10% FBS, (C) 2.1SHya in DMEM with 10% FBS, and (D) 2.1SHya only in DMEM into 35-mm tissue culture dish (NUNCLON) were incubated at 37 °C for 2 h under the 5% CO_2–95% air conditions, respectively, and washed with PBS (–) three times. Then, rOB cell suspensions in DMEM were added to four kinds of dishes (A–D). After 24 h-incubation, the cell appearances were observed as shown in (A–D). Magnification 100×.

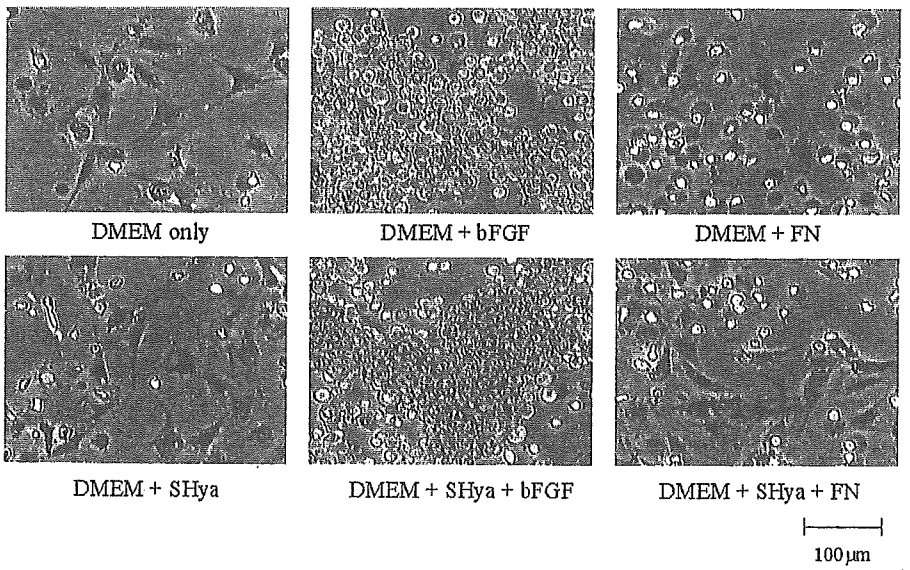

Fig. 9. Effect of serum component and 2.1SHya on cellular adhesion after 24 h. The cells were plated in serum free DMEM supplemented with FN, bFGF, and SHya, and incubated for 24 h at 37 °C with 5% CO_2.

the control or Hya addition. It is known that the osteoblast shifts to differentiation after it stops proliferation [30]. Recently, C-terminal Cx protein was found to suppress cell proliferation [31]. Then, we evaluated the effect of SHya on the ALPase activity of the initial differentiation marker for the osteoblast. The ALPase activity of rOB cells cultured with SHya was only expressed in the aggregation, when localization of the

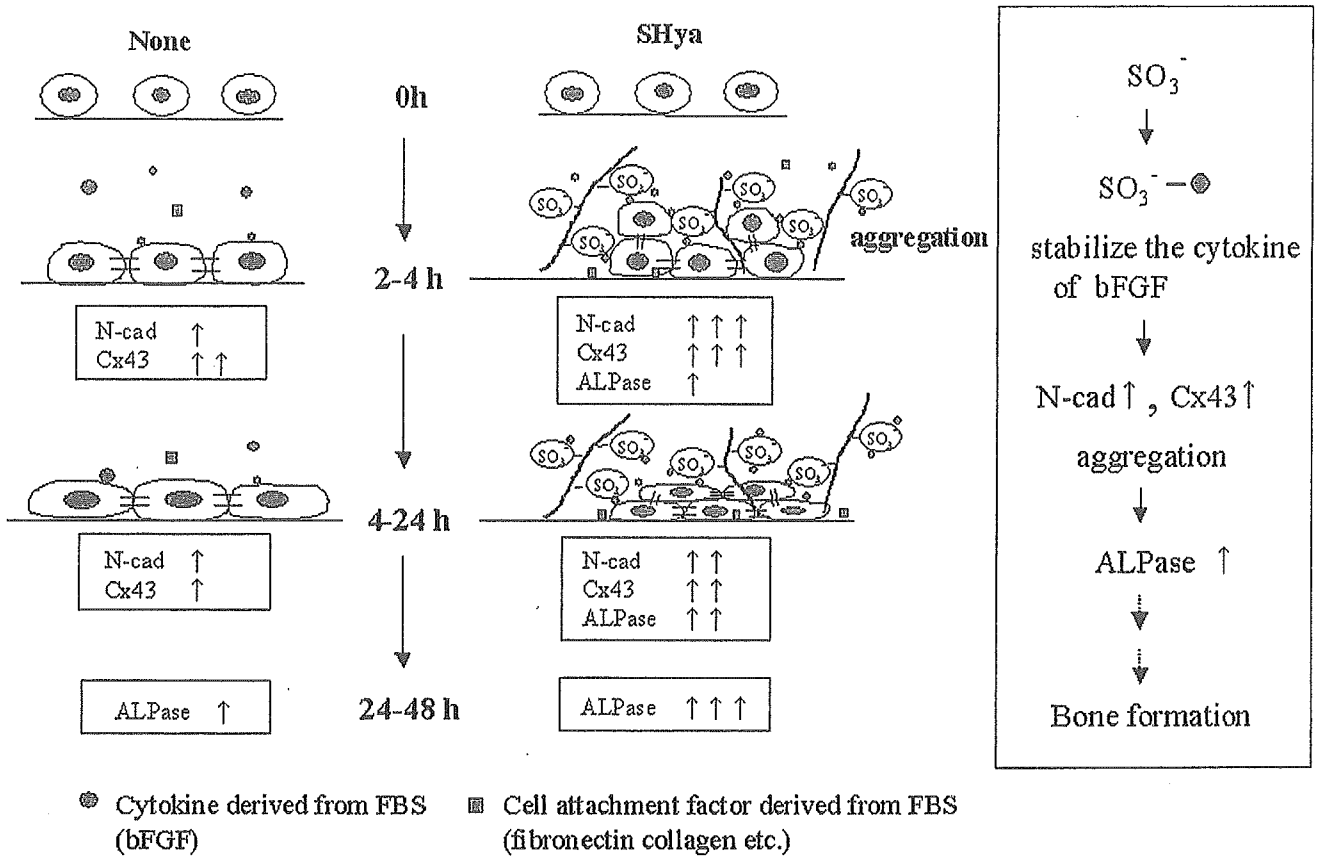

Fig. 10. SHya regulation of rat osteoblast cell differentiation.

ALPase activity was examined using the Azo staining method. The increased ratio and value of rOB cell ALPase activity with addition of SHya was higher than that of the control or Hya addition after 8 h. From the above results, the differentiation of rOB cells is promoted in aggregation, but the degree of proliferation is low as a whole. Therefore, it was shown that SHya controls rOB cell proliferation and differentiation, and that it especially promotes the differentiation. In this experiment, ALPase activity is enhanced while the expression of N-cad and Cx43 of rOB cells rises (Figs. 3, 6, and 7). Therefore, the expression of N-cad and Cx43 of rOB cells forming aggregations rises, and seems to promote the differentiation function, ALPase activity is enhanced with the aggregation formation of rOB cells.

In this experiment, 10% FBS was included in the culture medium. A serum of the usual 5–10% was included for the general culture medium used by the cell cultures of in vitro. The serum contained many components such as hormone, growth factor, cell adhesion molecule, and transportation protein [32]. Therefore, SHya interacted with the serum component, and it seemed to affect the cell. We examined the effect on the rOB cells by adding SHya to the serum-free medium. As the result, aggregations were not formed. However, when SHya coexisted with the serum, rOB cells formed aggregations. The interaction between the cell aggregation and the serum component such as FN, bFGF, and SHya was examined. In the case of FN, there was no effect on the cell aggregation. However, in the case of bFGF, cell aggregations were observed in both conditions with and without 2.1SHya. Therefore, it seems to relate the function of bFGF to the cell aggregation. N-cad expression of osteoblast by bFGF has been reported [22].

From these results, the effects of SHya on rOB cell function were not from the SHya alone; the data indicated that SHya affected rOB cell aggregation, proliferation, and differentiation by interacting with the serum component such as FGF and ECM (Fig. 10).

In conclusion, early expression of N-cad and Cx43 by SHya is the key to forming aggregations and enhancing the ALPase activity in rOB cells.

Acknowledgments

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BIOCOMPATIBLE BIOMATERIALS FOR THE HUMAN CHONDROCYTE DIFFERENTIATION ESTIMATED BY RT-PCR METHOD.

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Abstract

Biocompatibility of the biomaterials for the differentiation of the human articular chondrocytes were estimated by reverse transcription-polymerase chain reaction (RT-PCR). We used five biodegradable polymers for culturing with human articular chondrocytes. In addition to these five materials, we also estimated aquous type of fullerene, namely C60 dimalonic acid (C60DMA). Cultures were carried out using micromass culture method for 4 weeks. Collagen type II, aggrecan and connexin43 gene levels were estimated using RT-PCR methods. Among the biomaterials, Poly glycolic acid (PGA) showed the highest expression level of the collagen type II gene. On the contraty, C60DMA showed the lowest expression level among six kinds of test substances. In the case of the aggrecan gene, PGA also showed the highest levels, and C60DMA showed the lowest ones. However, the expression patterns of the connexin 43 gene were different from previous two genes. Using the multi regression analysis was carried out between differentiation and these three gene expression levels. There was a high correlation between cellular differentiation and three gene expression levels.

1. Introduction

Properties of degradation of scaffolds are the important character in the long-term success of a tissue-engineered cartilage construct. The biodegradable polymers hold the additional advantage that the cartilage tissue, with the biodegradation of the polymers, may gradually replace the space occupied by the scaffolds. Extensive studies have been carried out using bioreabsorbable materials. However, most of those studies used animal cells, whereas little information is available on the chondrogenic effects of these materials with human articular chondrocytes (HAC). The biocompatibility of the biodegradable polymers using human articular cartilage in a micromass culture system was studied. In the present in vitro micromass study, we investigated the biocompatibility of a synthetic biodegradable materials and a fullerene derivative of C60 dimalonic acid (C60DMA) as the indication of the cellular proliferation, differentiation and the expression level of 3 genes such as collagen type II, aggrecan and connexin43, estimated by RT-PCR method.

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2. Materials and Methods

Cell and Materials

Chondrocyte growth medium and HAC were commercially obtained from BioWhittaker, Inc. (Walkersville, MD, USA). Chondrocytes growth medium contains bovine insulin, basic fibroblast growth factor, insulin like growth factor-1, transferring, gentamicin sulfate and fetal bovine serum (5% v/v). PGA (Mw = 3,000) and PLGA (Mw = 5,000) were purchased from Nakalai Tesque Inc. (Kyoto, Japan) and, PGCL (Mw = 3,000) was from Taki Chemical Co (, Japan). P(LA-CL)25 Mw = (10,000) , PCL (Ti) (Mw = 130,000) and fullerene C60-dimalonic acid (C60 DMA) were synthesized in our laboratoty.

Cell culture

In vitro high-density micromass cultures of HAC were initiated by spotting $4x10^5$ cells in 20 μl of medium onto each well of 12-well microplates for tissue culture (Costar ® Type 3513, Corning Co. Ltd., NY, USA) and PCL(Ti) coated glass wells (diameter, 22mm). After two hours of cell spotting in a 5 % CO_2 incubator at 37°C, the wells were flooded with chondrocyte culture media (2 ml/well). Media were supplemented with DMSO (0.8 μl/ml), PGA (50 μg/ml), PGCL (50 μg/ml), PLGA (50 μg/ml), P(LA-CL)25 (50 μg/ml), and fullerene C60 DMA (60 μg/ml), respectively. HAC cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and the cultures were continued for 4 weeks.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource, International, Inc, Camarillo, CA) assay after 4 weeks of culture as previously described.[1]

Differentiation assay

Proteoglycans are typical contents of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage specific proteoglycans with alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described.[2]

RNA harvest

After the designated 4 weeks culture period, RNA was extracted from all matrices except PCL(Ti) matrix. For PCL(Ti) matrix, we did not have enough samples for RNA harvest as cells from 50 % of the cultured wells were detached over night following cell spotting. Total celluar RNA was extracted from cultured cells of four wells (for each material) in 0.5 ml Trizol reagent (Life Technologies, Inc., Frederick, MD, USA) according to manufacturer's instruction.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study was collagen type II and aggrecan. The gap junction protein gene of Cx43 was also studied. The single strand cDNA was prepared from 1 μg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After proper optimization of PCR condition, subsequent PCR was performed with 4 μg of cDNA in a 20 μl reaction mixture (10 x PCR buffer 2 μl,

dNTP 1.6 μl, forward and reverse, each primer 0.4 μl, Taq DNA polymerase 0.1 μl and rest of the amount of distilled water). The codon sequence used for the primer sets was as follows:

Collagen type II: forward 5'-GGCAATAGCAGGTTCACGTACA-3'
reverse 5'-CGATAACAGTCTTGCCCCACTT-3'

Aggrecan: forward 5'-TCGAGGACAGCGAGGCC-3'
reverse 5'-TCGAGGGTGTAGCGTGTAGAGA-3'.

Connexin 43 (Homo Sapiens):
forward 5'-ATGGGTGACTGGAGCGCCTTAGGCAAACTC-3'
reverse 5'- GACCTCGGCCTGATGACCTGGAGATCTAG -3'

The polymerization of GAPDH was accomplished by 25 cycles with the corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for the visualization of collagen type II and aggrecan and, on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH):

forward 5'-CCCATCACCATCTTCCAGGAGCGAGA-3'
reverse 5'-TGGCCAAGGTCATCCATGACAACTTTGG-3'.

3. Results

Cell proliferation assay

The cell proliferations of PGA, PGCL and PLGA were fairly parallel as that of control cell proliferation. The cell proliferation of P(LA-CL)25, PCL(Ti) and fullerene C-60 DMA were significantly inhibited as compared to control. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti) and fullerene C-60 were 101, 102, 104, 93, 84, and 93 %, respectively.

Proteoglycan synthesis

Intensity of alcian blue staining was found to be higher in PGA, PGCL and PLGA containing cultures than that was found with the control culture. Among the biomaterials, PGA caused a significant 3.1 fold increase of cell differentiation when compared to control ($p < 0.05$).

Extracellular matrix genes expression

RT-PCR analysis showed that all matrices consistently expressed collagen type II gene and PGA matrix had the strongest induction. Slight increase expressions of collagen type II gene were noted with PGCL and PLGA matrices. Expression of collagen type II gene in P(LA-CL)25 was faint and in fullerene C60 DMA was almost nil. PGA matrix showed the strongest induction of aggrecan gene. Aggrecan gene expressions were decreased in PLGA and P(LACL)25 matrices.

Expression of gap junction protein connexin 43 gene

PGA induced the highest level of Cx43 mRNA expression and moderate level of expression was noticed in PLGA treated culture. A faint expression in P(LA-CL)25 and almost nil expression in flurrene C60 DMA treated cultures were observed.

Multi-regression analysis

Using the multi regression analysis, correlation was investigated between the differentiation estimated by alcian blue method and three genes expression levels. There was a high correlation between the cellular differentiation and three gene expression (correlation coefficient is 0.96) (Fig.1). Especially, two kinds of expression levels of aggrecan, and connexin 43 genes, were found to be critical factors for estimating the extent of cellular differentiation of human articular chondrocytes(Fig. 1).

4. Discussion

During differentiation, chondrocytes secrete extracellular matrix (ECM) molecules characteristic of cartilage, such as type II collagen, aggrecan, and link protein, offering an environment that preserves the chondrocyte phenotype. Therefore, chondrocyte are defined both by their morphology and ability to produce these characteristic ECM. Collagen type II is regarded as the most important component among the ECM molecules. Previous study detected type II collagen as early as 7 days after beginning 3-D culture and at 21 days, the matrix of the entire aggregate contained type II collagen.[3] Among the ECM molecules, aggrecan is a major proteoglycan[4] and had been reported that in chick cartilage, aggrecan starts to be expressed at embryonic day 5 in limb rudiments, continues through the entire period of chondrocyte development, and remains a biochemical marker of the cartilage phenotype thereafter.[5] In this study, we have well demonstrated cell differentiation with the formation of cartilaginous nodules on culture plate, by alcian blue staining, which is commonly used for identification of cartilage, and by expression of ECM molecules collagen type II and aggrecan. The morphology after the designated culture period revealed that cells aggregated on the culture plate and resulted in the formation of cartilaginous nodules. The greatest cell differentiation, 3.1-fold increase of the controls was found in the sample treated with PGA. The potencies of cell differentiation after 4 weeks of culture from most to least were in the following order; PGA>> PLGA > PGCL > Cont. = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C60 DMA. The increased cell differentiation with PGA and PLGA matrices are in agreement with our previous findings in micromass culture system[1], however, in this study we have included the matrix genes expression of these materials. Results of the present study confirmed PGA and PLGA as useful scaffolding matrices for cartilage tissue engineering, and knowledge with other matrices will further contribute to develop improved cartilaginous constructs for future clinical implants. In this study, RT-PCR analysis showed that the mRNA level of x43 gene expression was consistent with the chondrogenic differentiation in the presence of different biomaterials. Our findings of Cx43 expression by chondrocytes are in agreement of previous study that reported expression of functional gap junctions by chondrocytes isolated from adult articular cartilage[6]. Gap junction mediated intercellular communication is critically involved in the development of cartilage during differentiation[7].

In this study, the data of cell differentiation by alcian blue and, observed expression of collagen type II, aggrecan and Cx43 suggest that the process of cell differentiation might be due to the interconnection of cells by means of gap junction along with other molecular mechanism. However, the specific association of gap junction in the process of chondrogenic differentiation and the cell signaling processes remains unexplored. Future studies are required to analyze the specific role that the gap junction proteins have in chondrocyte differentiation.

5. Acknowledgement

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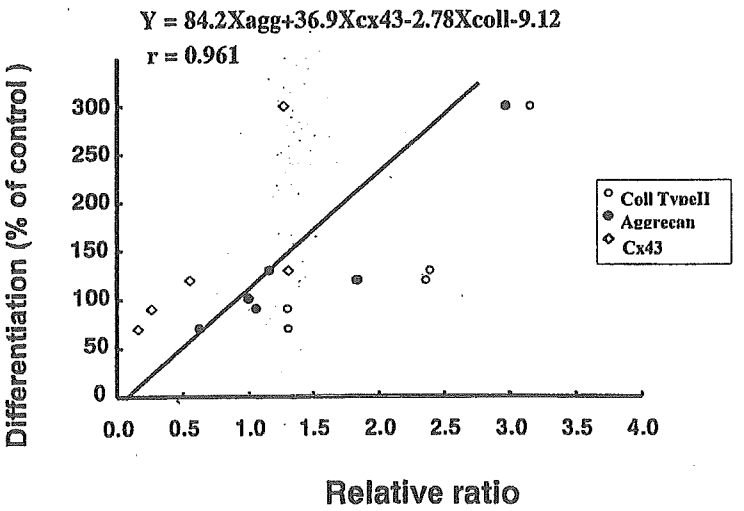

Fig.1.Relationship between the differentiation and the expression levels of three genes of collagen typeII, aggrecan and connexin43 using multi-regression analysis.

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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′-ATGAACTGGACAGGTTTGTACACCTT GCTC-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

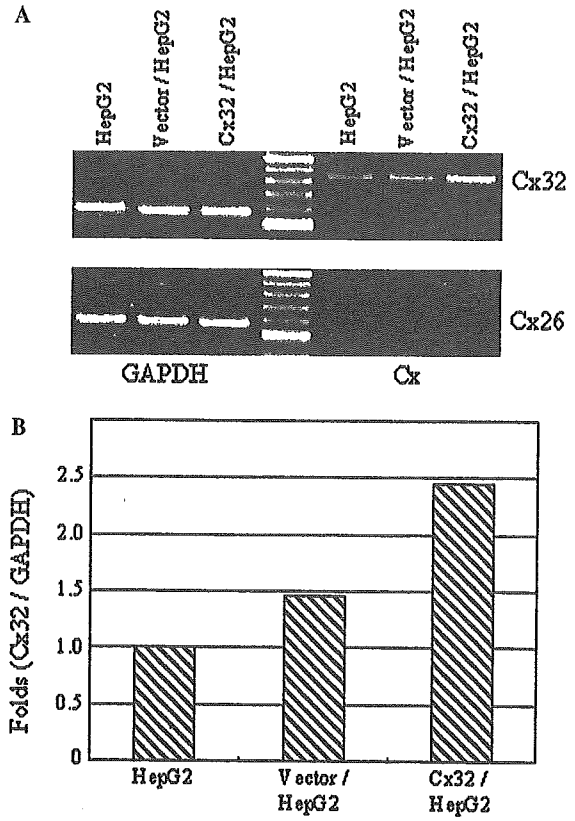

Fig. 1. Establishment of Cx32 and vector transfected HepG2. (A) RT-PCR analysis of Cx32 and Cx26 gene expression in HepG2, vector-transfected cells (Vector/HepG2), and Cx32 gene transfected cells (Cx32/HepG2). (B) Image assay of Cx32 gene expression in RT-PCR. Relative densities were standardized to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

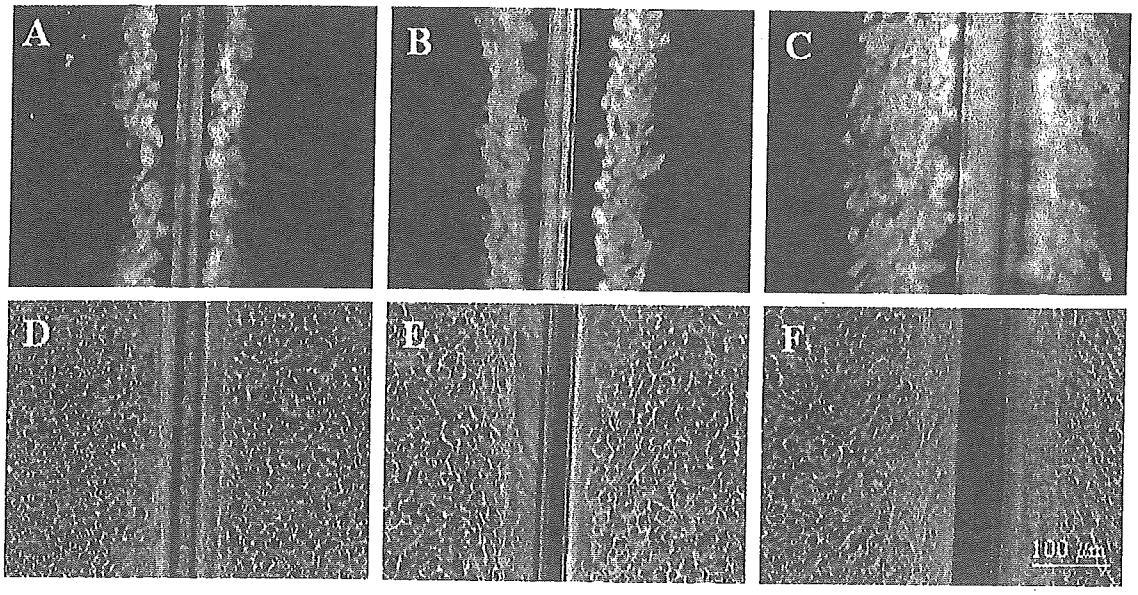

Fig. 2. Fluorescent (A–C) and phase-contrast (D-F) photographs of HepG2 (A and D), vector-transfected HepG2 (Vector/HepG2) (B and E), and Cx32-transfected HepG2 (Cx32/HepG2) (C and F) in the assay of scrape loading and dye transfer (SLDT), same scale in (A–F).

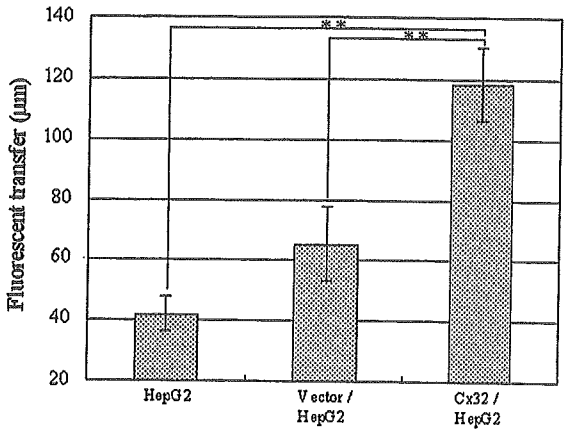

Fig. 3. Functional GJIC in HepG2, vector-transfected HepG2 (Vector/HepG2), and Cx32-transfected HepG2 (Cx32/HepG2) measured by the SLDT method at the seventh day of culture. Values are expressed as means from 20 determinations (**$P < 0.01$).

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 GIJC 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

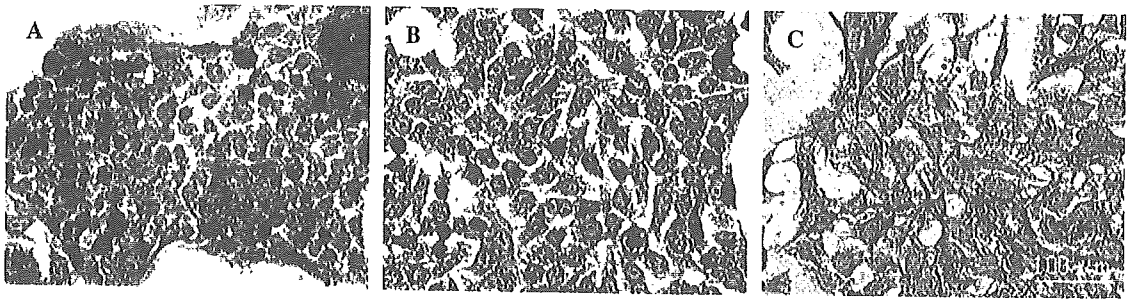

Fig. 4. Localization of Cx32 protein in HepG2 (A), vector-transfected HepG2 (B), and Cx32-transfected HepG2 (C). More gap junction plaques were detected in Cx32 gene transfected cells (arrow) than HepG2 and empty vector transfected cells. Immunocytochemical staining of Cx32 protein was performed with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

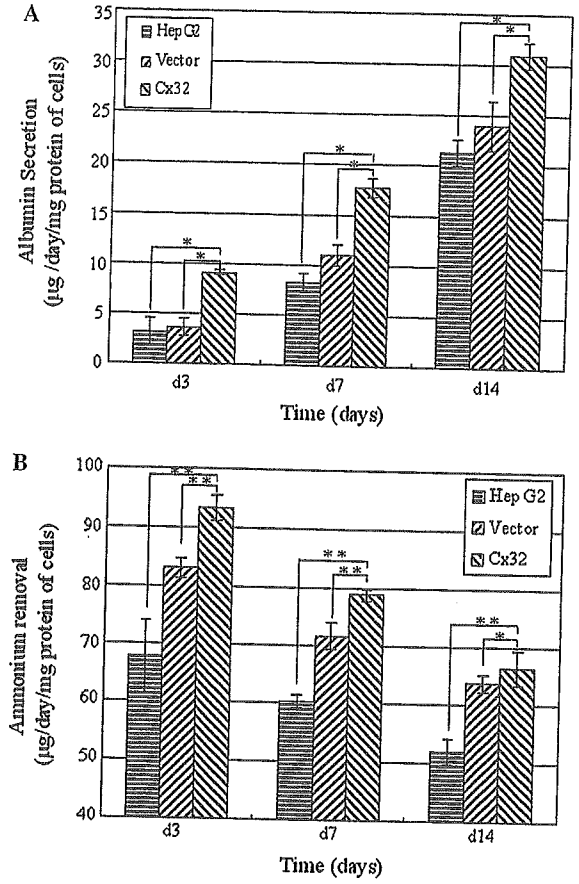

Fig. 5. Liver functions of albumin secretion (A) and ammonia removal (B) of HepG2, vector-transfected HepG2, and Cx32-transfected HepG2 examined on the 3rd, 7th, and 14th day of culture (*$P < 0.05$, **$P < 0.01$).

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.

Acknowledgments

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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,

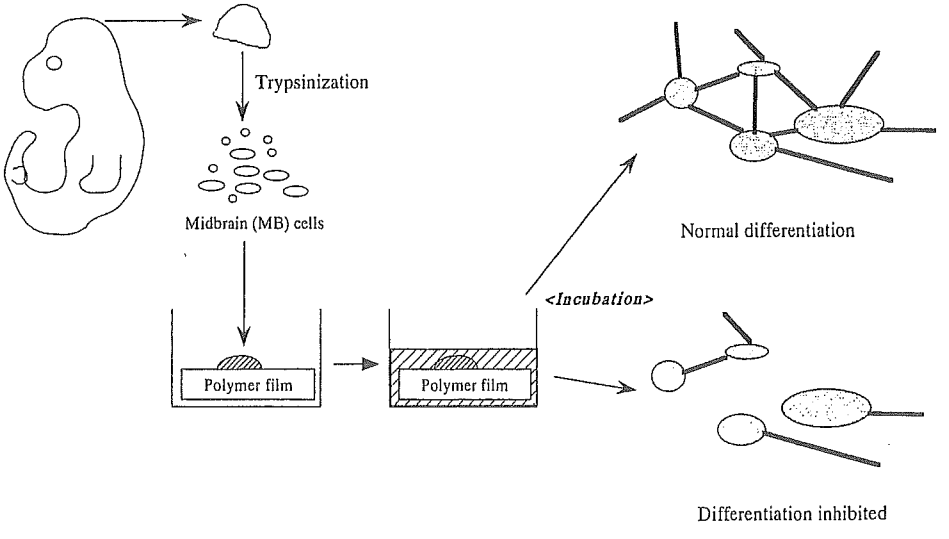

Figure 1. Schematic illustration of micromass culture system using MB cells.

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-

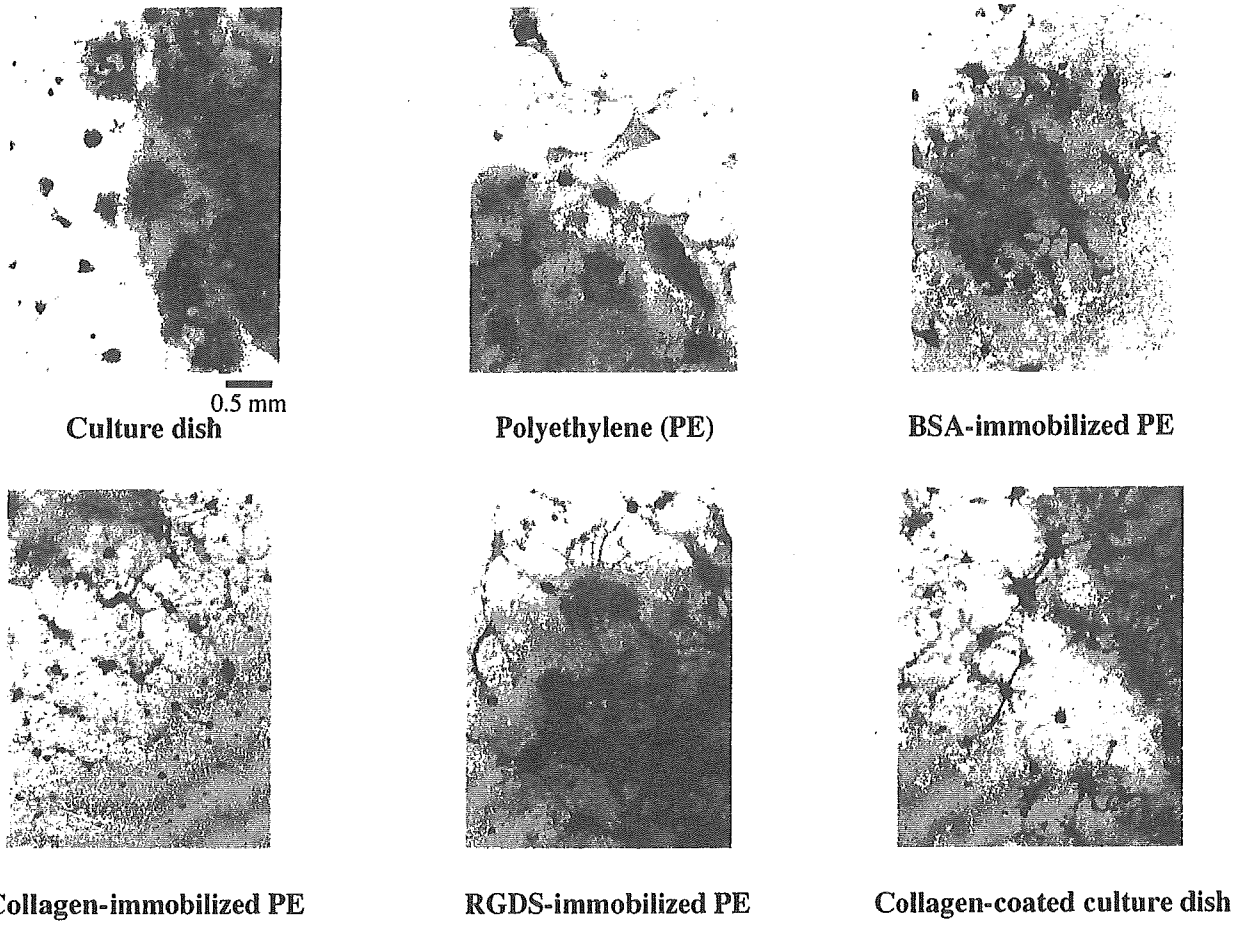

Figure 2. Light micrographs of Delafield's hematoxylin staining cultures of rat MB cells after incubation on various polyethylene surfaces.

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

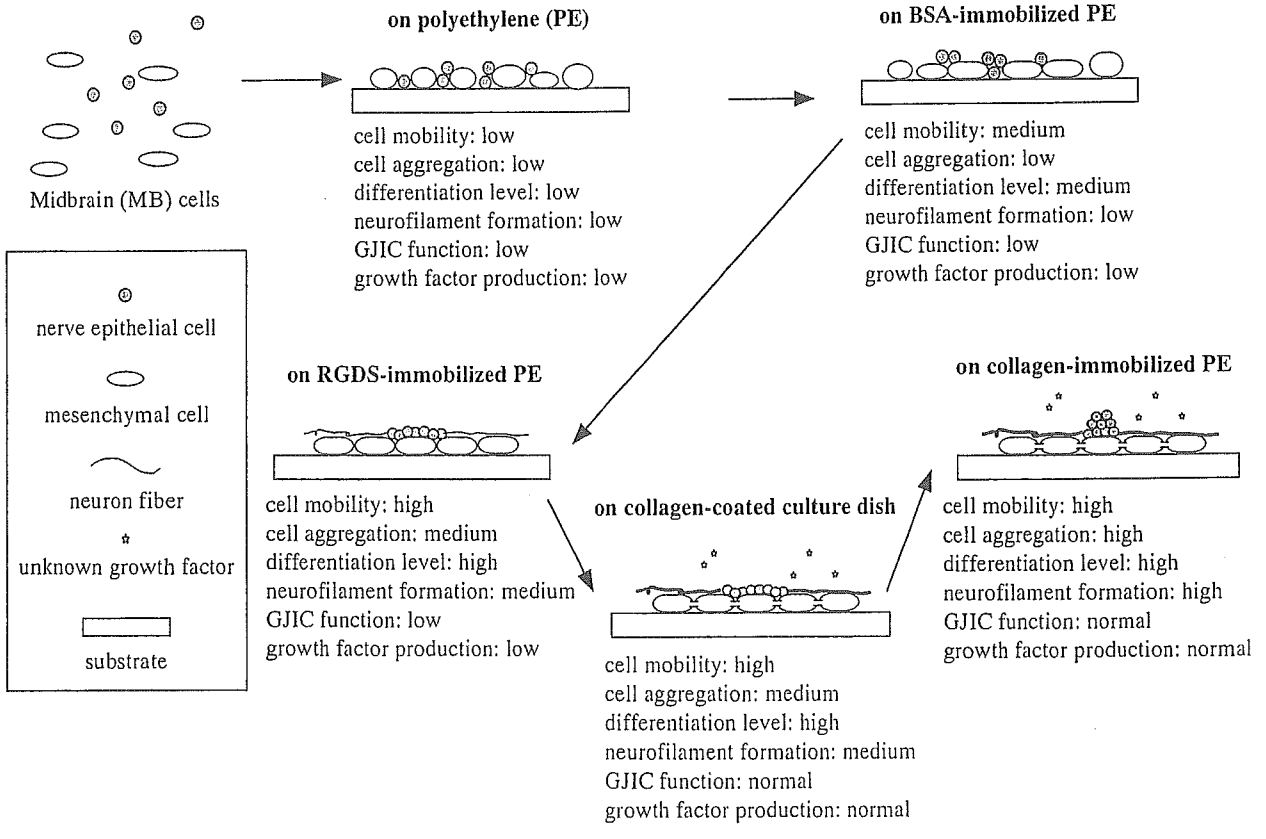

Figure 3. Schematic illustration of assumed differentiation mechanisms of MB nerve cells and mesenchymal cells after cultivation on various kinds of PE films.