1. INTRODUCTION

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

2. MATERIALS AND METHODS

2.1. ***NHDF Cell culture:*** The NHDF cells were obtained from Asahi Techno Glass (Tokyo, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5 % CO_2 atmosphere at 37°C.

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

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

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

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

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

2.6. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS (+) and the extent of dye migration length was measured using fluorescence microscope.

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

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

3. RESULTS

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

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

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

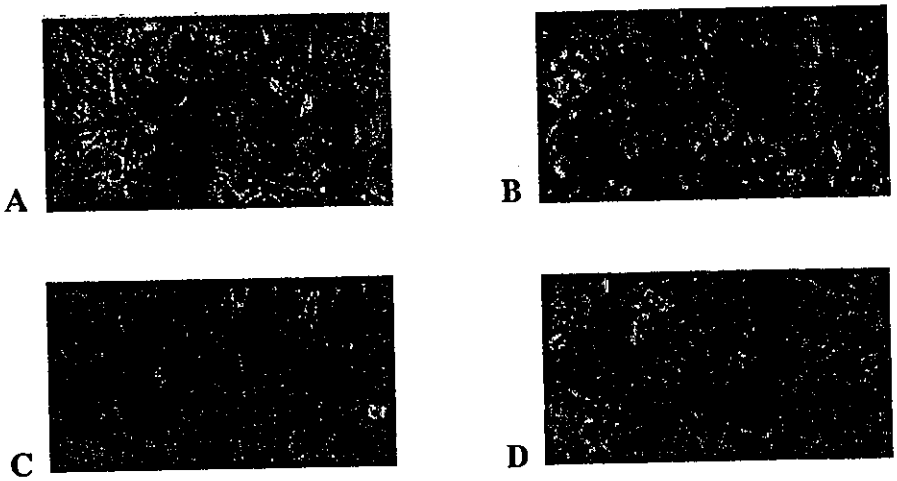

Figure 1 **Mice cells morphology. A: BAJB/cJ control, B: BALB/cJ implanted, C: SJL/J control and D: SJL/J implanted.**

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

4. DISCUSSION

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

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

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A novel function of N-cadherin and Connexin43: marked enhancement of alkaline phosphatase activity in rat calvarial osteoblast exposed to sulfated hyaluronan

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Abstract

In this study, we examined the interaction of the osteoblast which forms bone and sulfated hyaluronan (SHya). For the purpose of the creation of a new functional polysaccharide, we introduced a sulfate group in hyaluronan (Hya) of high molecular weight, and SHya of high molecular weight could be obtained for the first time. When rat calvarial osteoblast (rOB) cells were cultured with a high concentration of SHya, they formed aggregated spheroids after 4 h and the spheroids grew to about 200 μm after 24 h. We examined the expression of cell adhesion molecules in order to clarify the mechanism of aggregate formation. The N-cadherin (N-cad) and Connexin43 (Cx43) expression level of rOB cells cultured with SHya remarkably increased after 2 h. A difference in the expression of Integrin β1 (Intβ1) could not be observed between the SHya addition and control group. The alkaline phosphatase (ALPase) activity of rOB cells cultured with SHya after 8 h was significantly enhanced in comparison with control. Therefore, the sulfate group of SHya seems to enhance expression of cell adhesion protein such as N-cad and Cx43, resulting in aggregate formation and further remarkable induction of the ALPase activity of rOB cells.

Keywords: Sulfated hyaluronan; Osteoblast; Aggregation; N-cadherin; Connexin; ALPase activity

It is reported that the extracellular matrix (ECM) provides positional and environmental information essential for tissue function [1]. ECMs are complex, consisting of several different classes of molecules that may regulate modeling and remodeling [2]. Sulfated polysaccharides, such as heparan sulfate (HS) or heparin (Hep), stabilize fibroblast growth factor (FGF) and transforming growth factor β (TGF-β) in an active conformation, protect them against pH, thermal, and proteolytic degradations, and strongly potentiate their mitogenic activity in many cell types. Growth factors play a key role in the process of bone repair [3,4]. However, when the size of the defect is large, growth factor alone is not enough for bone repair. One promising way of promoting bone repair is to use cell scaffold, such as collagen [5]. However, there are problems, such as the antigenicity on the proteins. Therefore, we tried the regeneration of the bone using biocompatibility polysaccharides. Hyaluronan (Hya) has by far the highest molecular weight of the glycosaminoglycans (GAGs) and is thought to facilitate cell migration, adhesion, proliferation, and tissue repair [6].

Then, we synthesized sulfated hyaluronan (SHya) with different degrees of sulfation. We examined the effect of SHya on the cell function of rOB cells.

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Materials and methods

Sulfated hyaluronan. On the sulfation of the polysaccharide, various methods are reported [7–10]. However, the sugar chain is easily cut off under reaction and the molecular weight lowers. Therefore, a method using sulfur trioxide (SO_3) complex was developed to prevent the lowering of the molecular weight [11–13]. The molecular weight simply lowers on Hya by acid and heating. Then, the synthesis was carried out using dimethylformamide (DMF)–SO_3 complex and trimethylamine (TMA)–SO_3 complex. Hya derivatives with different sulfation degrees can be obtained by changing the amount of DMF–SO_3 complex and TMA–SO_3 complex.

Dimethylformamide–SO_3 complex. Ten percent Hya150 (molecular weight, 1.5×10^6) solution in *N,N*-dimethylformamide (DMF) (WAKO Pure Chemical Industries) was mixed with DMF–SO_3 complex [14] and stirred for 14 h at 0 °C. The reaction mixture was then diluted, neutralized, and precipitated by adding to a large quantity of acetone. The precipitate was dissolved in distilled water again and dialyzed against distilled water.

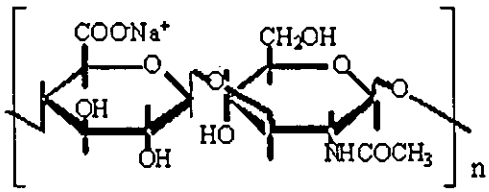
Trimethylamine–SO_3 complex. Ten percent Hya150 solution in DMF was mixed with TMA–SO_3 complex (Aldrich Chemical) and stirred for 48 h at 60 °C. SHya was obtained after the reaction by the method equal to the above-mentioned DMF–SO_3 complex method.

The degree of substitution (DS) of SHya was 1.2, 2.1, and 3.4 as determined by the chelate titration method [15]. Moreover, the effectiveness of sulfation was also demonstrated by FT-IR analysis. The IR spectrum of SHya exhibited two absorption bands at 1240 and 820 cm^{-1} due to the S=O and SO_3^- stretching, respectively. Characteristics of SHya are summarized in Table 1 and chemical structures are illustrated in Fig. 1. The number, which is at the end of the compound's name, indicates MW [$\times 10^4$] and the subscript shows the DS.

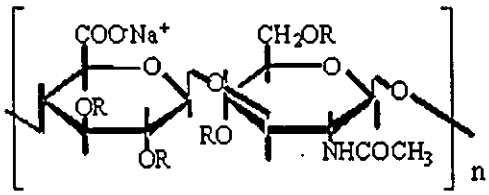
Cell culture. The rOB cells were isolated and cultured using the method described by Hamano et al [16]. rOB cells were cultured in a sterile tissue culture dish (NUNCLON) with the use of Dulbecco's modified Eagle's medium (DMEM, Nissui-seiyaku) supplemented with 10% fetal bovin serum (FBS, Gibco). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C. The cells were plated in 24-well tissue culture plates (NUNCLON) or 100 mm ϕ tissue culture dish (NUNCLON) at an initial density of 5×10^4 cells/cm^2 for study of the effects of Hya and SHya on cell function. The cells were subconfluent after 2–3 days of culture and confluent after 3–4 days.

Table 1
Characteristics of polysaccharides

Polysaccharides	Number of sulfate groups per two saccharide rings	MW ($\times 10^4$)
Hya	0	30
1.2 SHya	1.2	55
2.1 SHya	2.1	20
3.4 SHya	3.4	5



Hyaluronan (Hya)



Sulfated hyaluronan (SHya)

R= SO_3Na or H

Fig. 1. Structure of hyaluronan and sulfated hyaluronan.

Western blotting analysis. Immunoblots of N-cadherin (N-cad), Integrin β1 (Intβ1), and Connexin43 (Cx43) were performed according to the method of Matsuda et al. [17]. rOB cells were plated in 100 mm ϕ dishes. The cells were incubated with SHya for different time intervals as indicated in the results, washed with phosphate-buffered saline (PBS (−)), and lysed for 30 min at 4 °C with RIPA buffer. After sonicating the lysates for 30 s using a sonicator, their protein concentrations were determined using DC protein assay (Bio-Rad Laboratories). The lysate was mixed with equal volumes of Laemmli sample buffer, and proteins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes (OSMONICS). After blocking with 3% nonfat dried milk in Tris-buffered saline with Tween 20 buffer, the membranes were incubated successively with a primary antibody, followed by incubation with antimouse antibodies conjugated with ALP, and detection with ALP detection reagent (Gibco). Primary antibodies used include those recognizing N-cad, Intβ1, and Cx43. All antibodies were monoclonal mouse antibodies and were obtained from BD Transduction Laboratories.

Preparation of cell lysate for assay. Cell lysates were prepared according to the method of Hamano et al. [16]. After removal of the culture medium from the dishes, cells were washed three times with PBS (−). One milliliter of PBS (−) containing 0.04% Nonidet P-40 (Nacalai tesque) was poured into the dishes and incubated at 37 °C for 10 min. The suspension was homogenated with an ultrasonic disrupter (BH-200P, TOMY SEIKO) and centrifuged at 1000 rpm for 10 min at 4 °C. These cell lysates were used as sample solutions for the measurements of protein content and ALPase activity.

Protein content. Total protein content of cell lysate was measured by the BIO-RAD protein assay method (Protein assay, Bio-Rad Laboratories) and absorbance at 595 nm was measured using an ELISA reader (Bio-Rad Laboratories), using bovine serum albumin (WAKO Pure Chemical Industries) as reference standard.

Alkaline phosphatase activity. Alkaline phosphatase (ALPase) activity was determined by the modification of the methods of Hamano et al. [16] and Lowry et al. [18]. The reaction mixture consisted of 0.1 ml cell lysate and 0.4 ml of 16 mM *p*-nitrophenylphosphate disodium salt hexahydrate (WAKO Pure Chemical Industries). The solution was incubated at 37 °C for 30 min. The enzymatic reaction was stopped by adding 0.5 ml of 0.5 N NaOH and absorbance at 410 nm of *p*-nitrophenol liberate was measured. The enzyme activity was expressed in units/mg of protein, where 1 U corresponded to 1 nmol of *p*-nitrophenol liberate per 30 min at 37 °C. For determination of the localization of the ALPase activity, cells were rinsed with PBS (−) and fixed with 10% formalin (pH 7.4) overnight at 4 °C. These fixed dishes were rinsed three times with distilled water and Azo staining solution (5 mg naphthol AS-BI phosphoric acid sodium salt (FLUKA) in 10 ml of 0.05 M 2-amino-2-methyl-1,3-propandiol (WAKO Pure Chemical Industries) buffer (pH 9.8)) for 5 min at room temperature. Finally, they were washed three times with distilled water.

Culture conditions for estimating the interaction of serum and SHya. 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), and incubated for 2 h at 37 °C, respectively. After the incubation, these dishes were washed up with PBS (−) three times. rOB cells were suspended in DMEM without serum, the cell suspensions were added into these dishes, and cell adhesion and morphological change were examined after 24 h-incubation.

Interaction of serum components and SHya. The cells were plated in serum free DMEM supplemented with fibronectin (FN), basic FGF

(bFGF), and SHya. Cells in culture were incubated at 37 °C for 24 h with 5% CO_2.

Results

Fig. 2 shows the morphologies of the attachment of rOB cells cultured with four different concentrations of 2.1SHya after 24 h. rOB cells treated with high concentrations (0.25 and 0.5 mg/ml) of 2.1SHya formed large aggregations. Western blotting was used to examine the effect of 2.1SHya on adhesion protein expression in rOB cells. The cultures were washed with cold PBS (−) and protein samples were collected by the addition of a lysis buffer. As shown in Fig. 3A, the control time-dependently increased protein levels of N-cad, Intβ1 after incubation with rOB cells for 24 h. The time-dependence of 2.1SHya stimulation of N-cad is shown in Fig. 3B. This response was considerably earlier than that observed for the control, peaking 2–6 h after 2.1SHya addition (Fig. 3C). Expression of Intβ1 was not observed in great difference for the 2.1SHya addition and control. Cx43 expression level in the 2.1SHya addition reached a peak at 2–4 h, and increase in some expression levels of protein was observed in comparison with the control (Fig. 3C). N-cadherin in Fig. 4 shows the morphologies of the attachment of rOB cells treated with different DS SHya and Hya after 24 h. Cell aggregations were formed in the case of high DS SHya (2.1SHya, 3.4SHya). In the meantime, with low DS 1.2SHya or nonsulfated Hya, aggregations were not formed. However, when 1.2SHya was added in high density, rOB formed aggregations. Fig. 5 shows rOB cell proliferation in the presence of SHya and Hya. In the presence of 2.1SHya, cell proliferation was suppressed after seeding 48 h. However, rOB cells treated with 2.1SHya gradually proliferated afterwards and it reached confluence after 120 h. Hya showed similar trends in the control (TCD). Fig. 6 shows photographs of the Azo staining used for the determination of ALPase activity localization on rOB cell monolayers and aggregates cultured for 24 h. The staining also immaturely dyed the central part of the aggregation observed in the 2.1SHya. The rOB cells in TCD and Hya did not stain with Azo staining. The ALPase activity was only expressed in the aggregates. Compared with the control and Hya, 2.1SHya also time-dependently enhanced the ALPase activity of rOB cells when examined at a concentration of 0.5 mg/ml (Fig. 7). The effect of the existence of serum component and 2.1SHya on the formation of aggregation of rOB cells was examined (Fig. 8). rOB cells did not form aggregations without 2.1SHya in the case of the serum-free medium (Fig. 8D). The adherent cell number increased when it

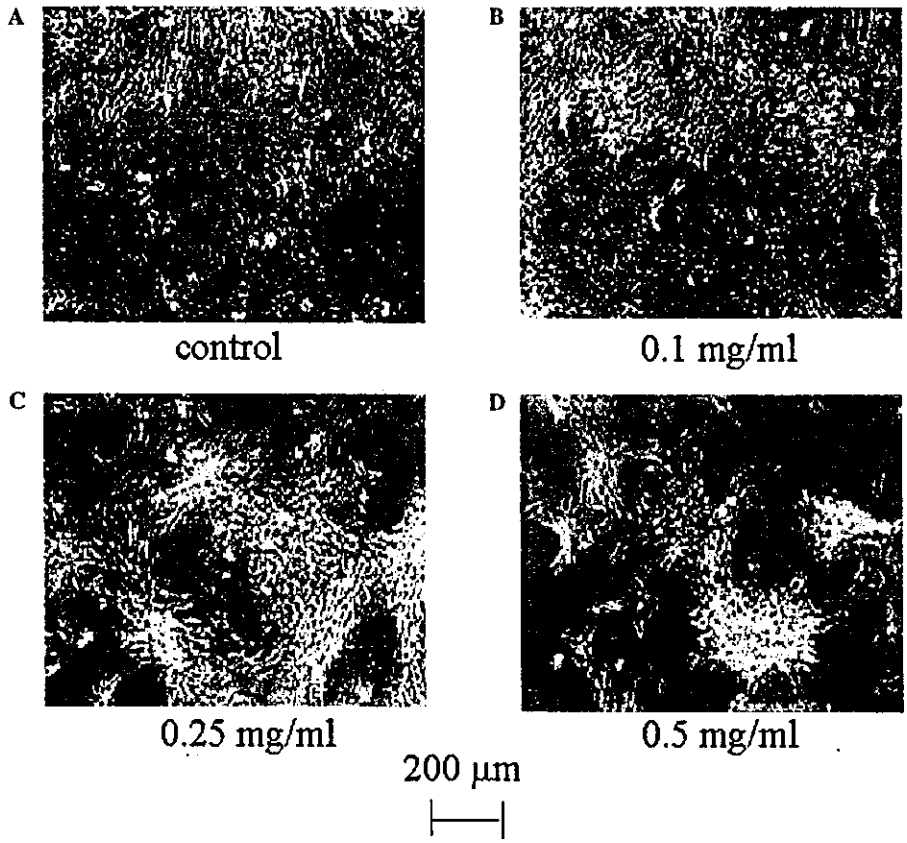


Fig. 2. Relationship between 2.1SHya concentration and rOB cell adhesion after 24 h. rOB cells were treated with various concentrations of 2.1SHya. (A) Control. (B) 0.1 mg/ml of 2.1SHya. (C) 0.25 mg/ml of 2.1SHya. (D) 0.5 mg/ml of 2.1SHya. Phase contrast micrographs. Scale bar 200 μm.

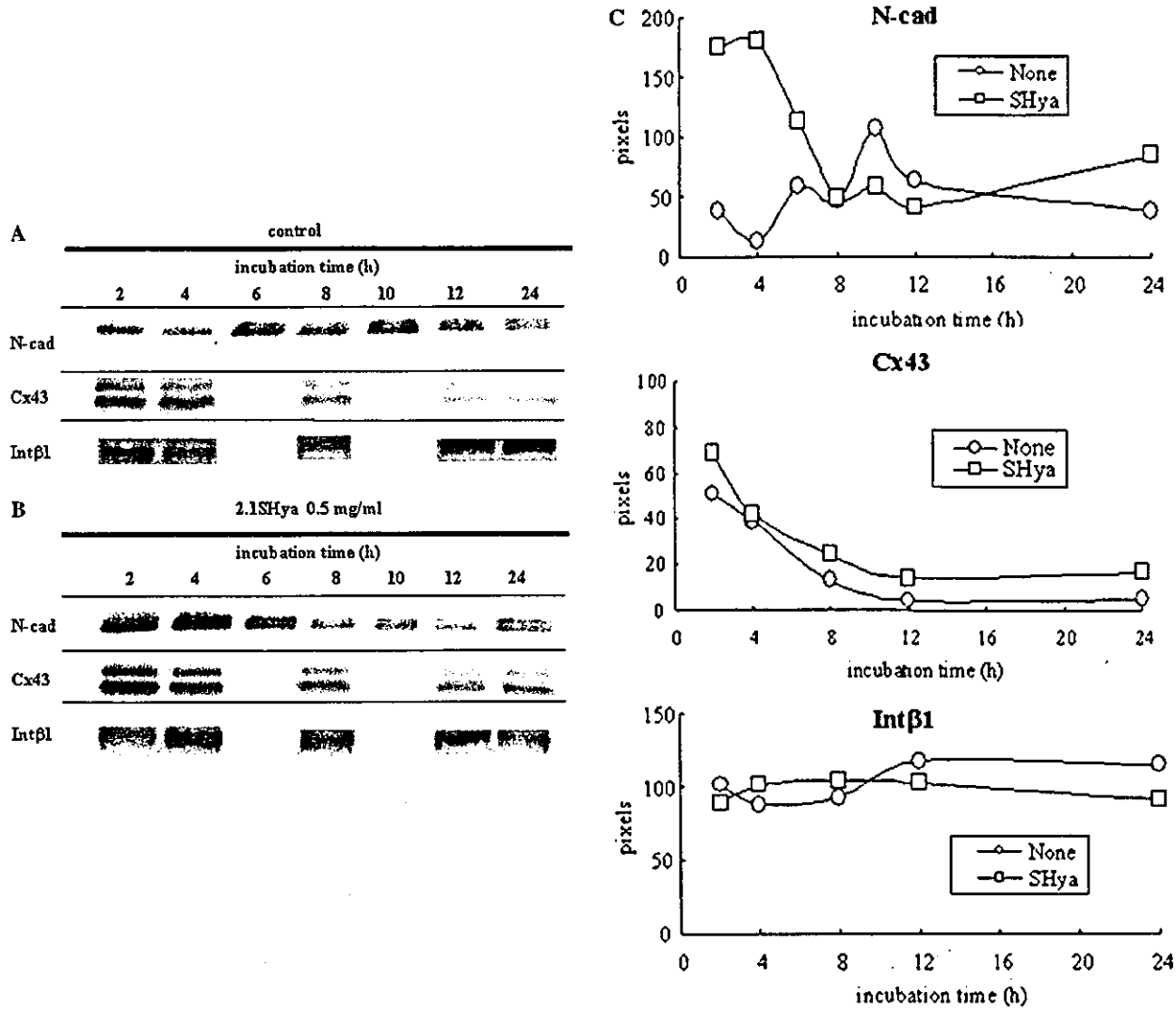


Fig. 3. Effect of SHya on adhesion protein expression in rOB cells. rOB cells were incubated with 2.1SHya for the times shown. Cells were lysed and proteins were separated by SDS–PAGE followed by Western blotting. (A) Without 2.1SHya (B) with 2.1SHya (C) quantification of band intensities was measured by NIH images.

was incubated in the culture medium including the serum (Fig. 8B) in comparison with the serum-free system. However, when SHya coexisted with the serum, rOB cells formed aggregations (Fig. 8C). rOB cells were seeded onto the plates in the presence or absence of FN and bFGF of added SHya for the study of effects of serum protein and SHya on cell aggregation (Fig. 9). bFGF was shown to form aggregation in rOB cells but not in the case of FN addition. Furthermore, when SHya was added with the bFGF, the cell aggregation was increased by the addition of SHya under the presence of bFGF.

Discussion

The aim of this study was to elucidate the mechanism of the enhancement of ALPase activity induced by the high molecular weight of sulfated polysaccharides. Hep, HS, and Hya are common components of the ECM in most tissues [19]. It is reported that sulfated polysaccharides like Hep/HS are the major FGF, TGF-β, and bone morphogenetic protein (BMP)-binding molecules in the ECM [20]. However, the molecular weights of Hep/HS and chondroitin sulfate (Chs) are lower than Hya [21]. Therefore, we synthesized SHya with varying DS and high molecular weight in order to obtain a high molecular weight of sulfated polysaccharides. Hya is easily decomposed in heat and acid [19]. Therefore, by the change of type and quantity of the SO_3 complex, SHya of varying DS and high molecular weight was synthesized. In this study, we examined the effect of SHya on the initial differentiation marker of the osteoblast. As a result of examining the effect of SHya in rOB cells on cell morphology, the following fact became clear: rOB cells formed aggregations in over 3 mg/ml

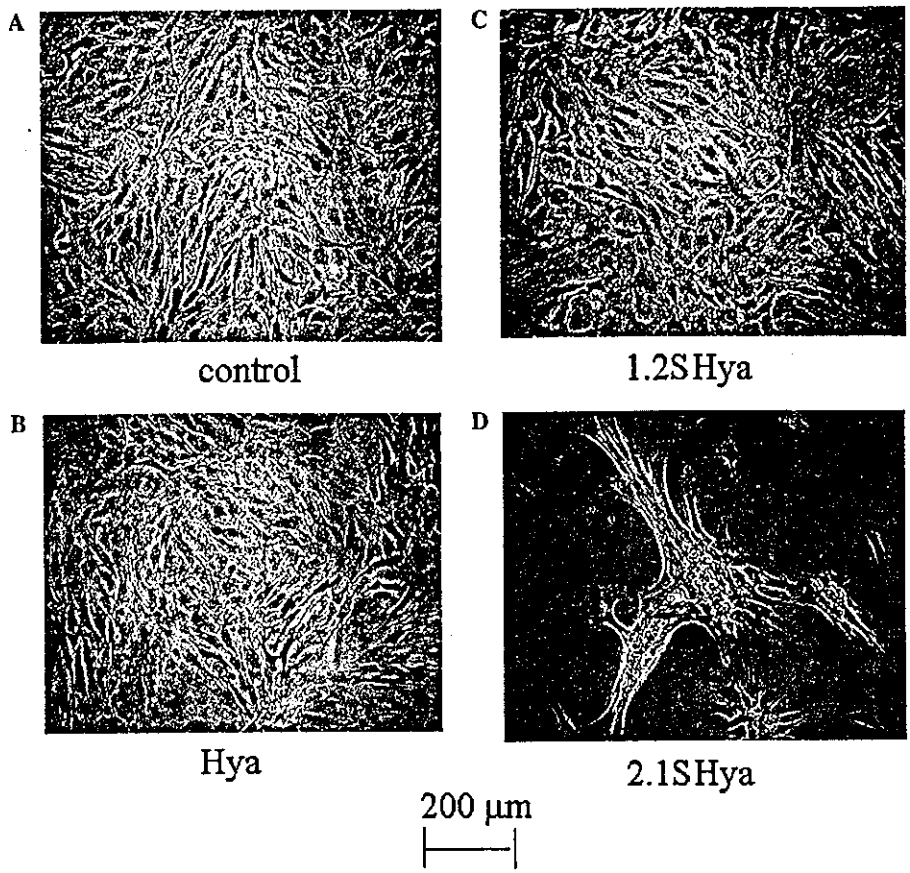


Fig. 4. Cell morphologies of rOB cells in the presence of 0.5 mg/ml Hya and SHya after 24 h. rOB cells were treated with Hya and varying DS of SHya. (A) Control, (B) Hya, (C) 1.2SHya, and (D) 3.4SHya.

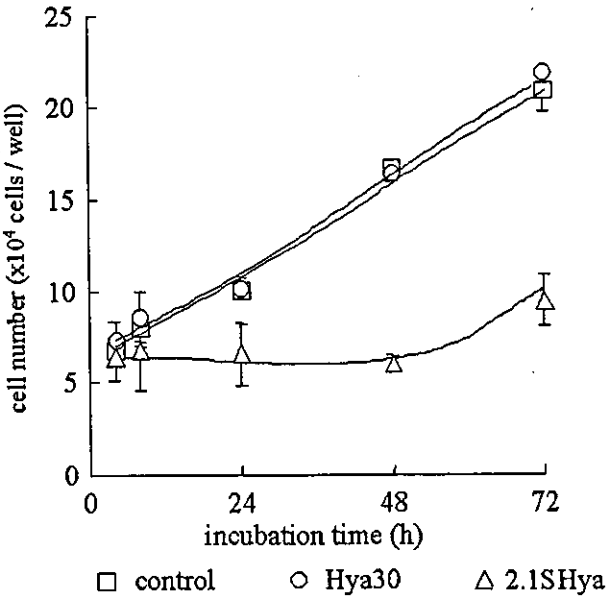
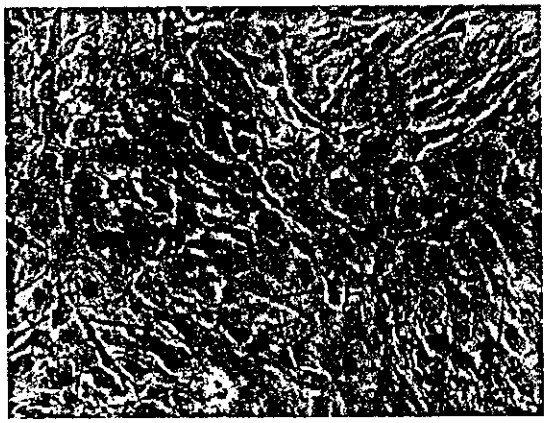


Fig. 5. Effect of 0.5 mg/ml Hya and SHya on the proliferation of rOB cells. rOB cells were treated with Hya and 2.1SHya for 72 h. The proliferation of rOB cells treated with Hya and 2.1SHya was determined. Values are means ± SD for four dishes.

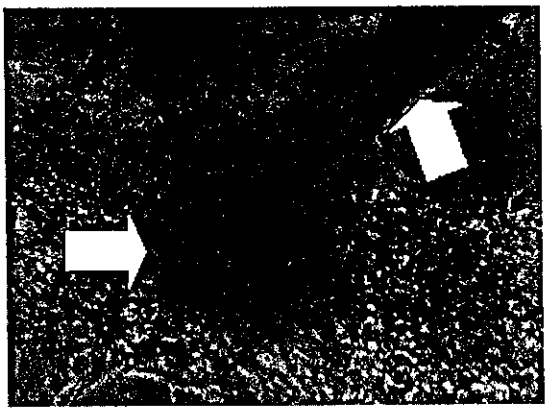
concentration in the case of SHya of low DS (1.2SHya) and in over 0.25 mg/ml concentration in the case of SHya of high DS (2.1SHya, 3.4SHya). However, rOB cells cultured with Hya without the sulfate group did not form aggregations (data not shown). Also, aggregations were not formed when Hep and Chs were added. After the SHya addition, rOB cells began to form aggregations after 4 h and large aggregations were formed after 24 h. Therefore, by introducing a sulfate group into the hyaluronan, rOB cells formed aggregations.

Cell–cell contacts and communication between bone cells are essential for coordinated bone development and remodeling. Cell–cell adhesion mediated by the cadherin superfamily plays an important role in osteogenesis. Cadherins play essential roles in the regulation of several physiological processes such as cell migration, proliferation, and differentiation [22]. Tsutsumimoto et al. [23] reported that the expression of N-cad is involved in the aggregate formation of MC3T3-E1. Also, integrins are the principle mediators of the molecular dialogue between a cell and its ECM environment such as collagen and fibronectin [24,25]. Osteoblasts express several integrin subunits and their presence may be important in

control



Hya



2.1SHya

Fig. 6. Appearance of Azo-stained cultures of rOB cells in the presence of 0.5 mg/ml Hya and SHya after 24 h. rOB cells were treated with Hya and 2.1SHya for 24 h. rOB cells were stained by the Azo stain method.

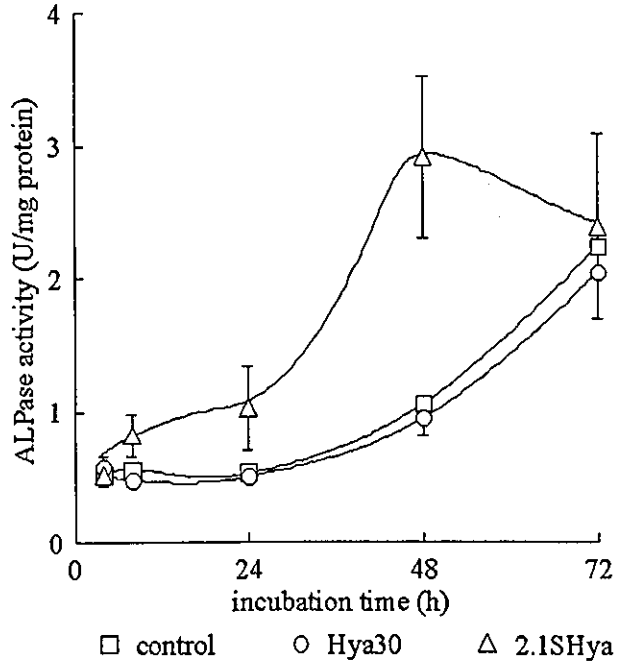


Fig. 7. Effect of 0.5 mg/ml Hya and SHya on the ALPase activity of rOB cells. rOB cells were treated with Hya and 2.1SHya for 72 h. The ALPase activity of rOB cells treated with Hya and 2.1SHya was determined. Values are means ± SD for four dishes.

regulating the response of these cells to the ECM, suggesting that integrin participates in the differentiation. By Western blotting, the expression of N-cad and Intβ1 proteins in osteoblasts was confirmed. In the presence of 2.1SHya, rOB cells increased protein levels of N-cad at early stages, but protein levels of Intβ1 were not observed in great difference between the 2.1SHya addition and control group. To clarify the roles of N-cad in SHya-induced cell aggregation, the effects of N-cad function-perturbing agents such as blocking antibodies were tested. This N-cad antibody was shown to inhibit cell–cell aggregation in rOB cells. These results confirm a direct involvement of N-cad in aggregation process (data not shown). Gap and adherens junctions are observed in osteoblast cell–cell contact [26,27]. Gap junctional intercellular communication (GJIC) is the key function by which cells exchange small molecules including signal molecules directly from the inside of a cell to neighboring cells. Gap junctions that are mediated by Cx have been well studied in osteoblasts. Among the Cx family, Cx43 is a major protein in osteoblasts [28]. By Western blotting, the expression of the Cx43 protein in these cells was confirmed. Cx43 expression level in the 2.1SHya addition reached a peak at 2–4 h, and the increase in expression level of protein was observed in comparison with the control. Some reports have proposed that cadherin is also involved in the regulation of the GJIC. This suggests that cadherin-mediated cell–cell adhesion is essential for GJIC and cadherin may also regulate GJIC in osteoblasts. Chiba et al. [29] demonstrated that Cx43 expression parallels ALPase activity and osteocalcin secretion in differentiating human osteoblastic cells. These data suggest that Cx43 expression contributes to osteoblastic differentiation.

Proliferation of rOB cells after aggregation formation was inhibited with the SHya addition more than with

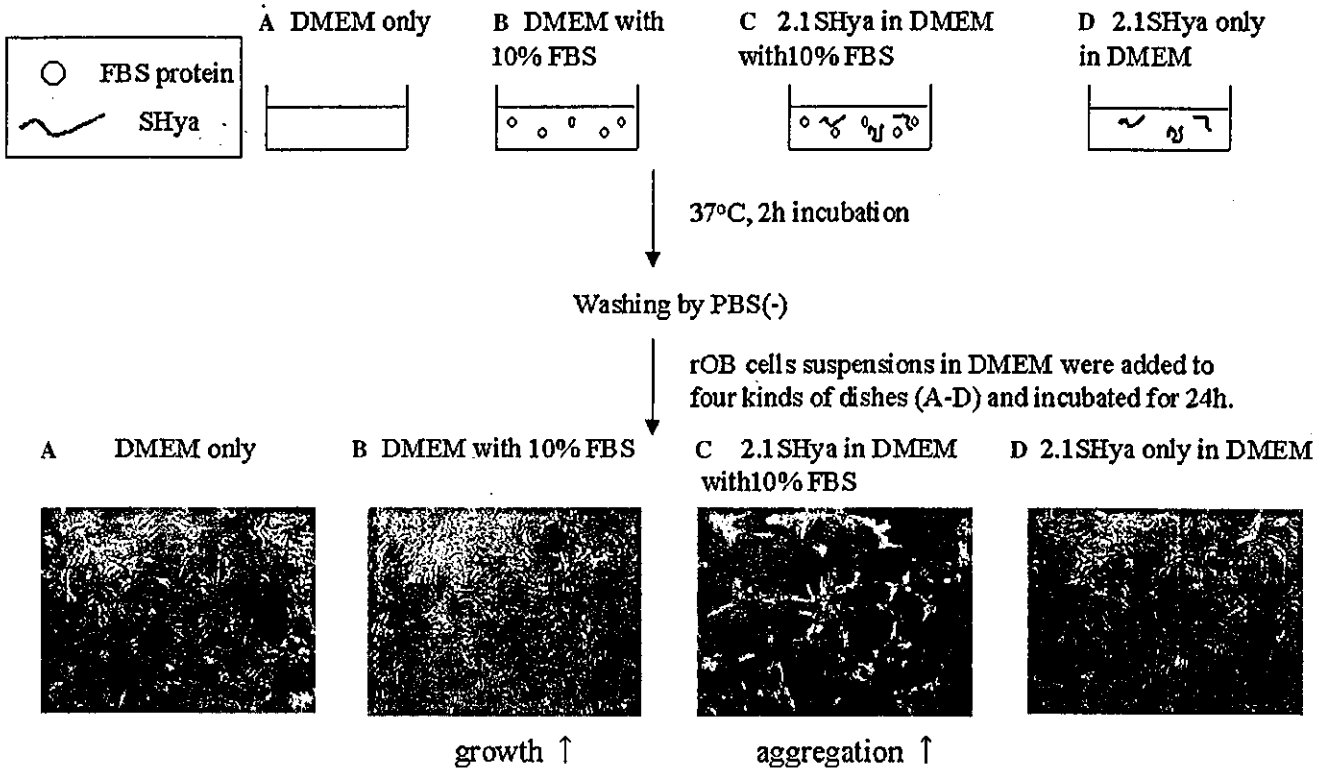


Fig. 8. Effect of FBS and 2.1SHya on cellular adhesion after 24 h. Four kinds of dishes were prepared as follows: (A) DMEM only, (B) DMEM with 10% FBS, (C) 2.1SHya in DMEM with 10% FBS, and (D) 2.1SHya only in DMEM into 35-mm tissue culture dish (NUNCLON) were incubated at 37 °C for 2 h under the 5% 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×.

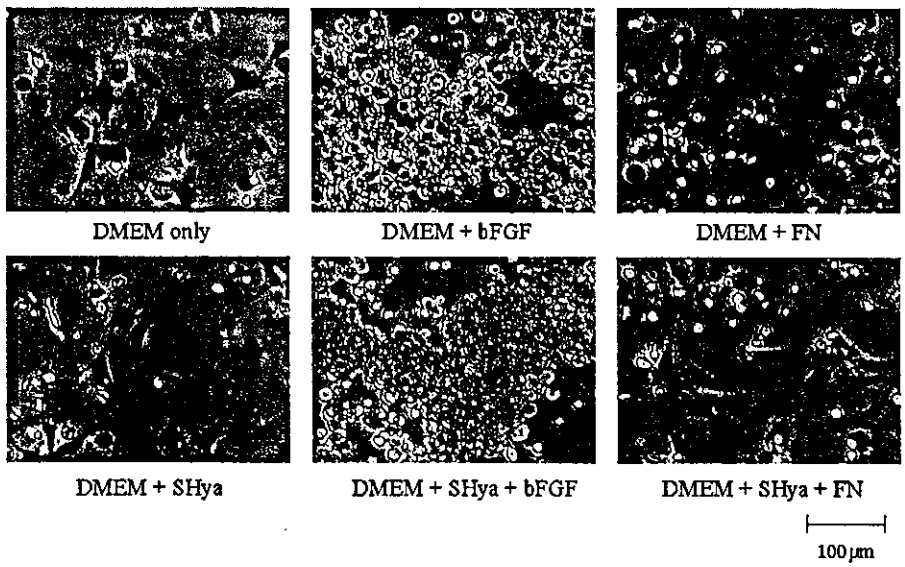


Fig. 9. Effect of serum component and 2.1SHya on cellular adhesion after 24 h. The cells were plated in serum free DMEM supplemented with FN, bFGF, and SHya, and incubated for 24 h at 37 °C with 5% CO_2.

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

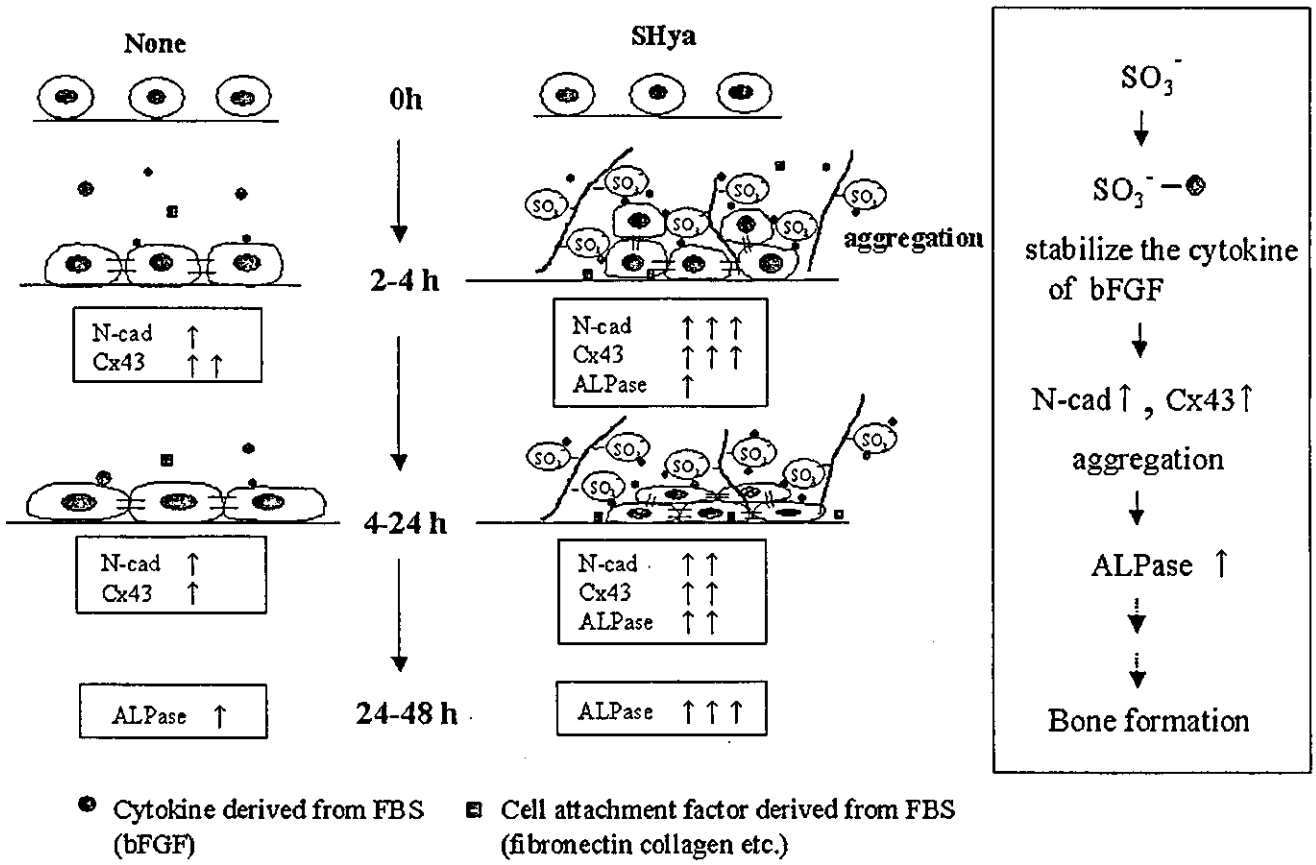


Fig. 10. SHya regulation of rat osteoblast cell differentiation.

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

We are grateful to the support of Health and Labour Sciences Research Grants, Research on Advanced Medical Technology, Ministry of Health, Labour and Welfare and Japan Health Sciences Foundation.

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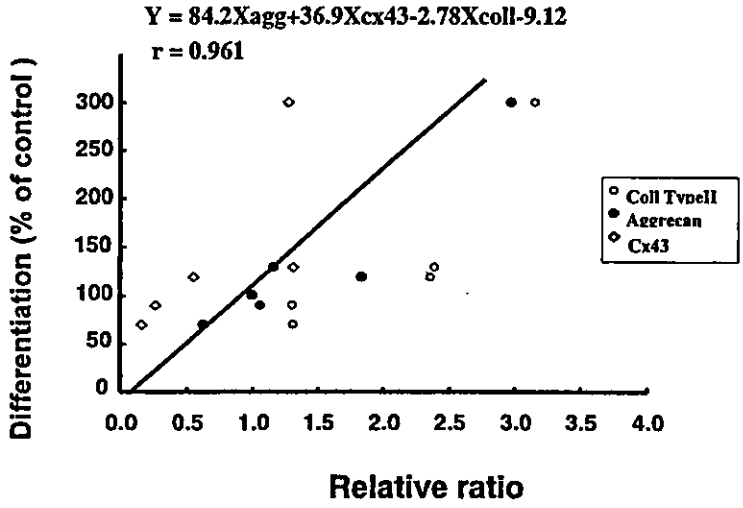

Fig.1.Relationship between the differentiation and the expression levels of three genes of collagen typeII, aggrecan and connexin43 using multi-regression analysis.

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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