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.

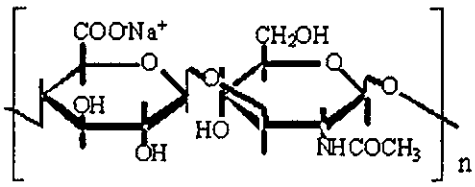
Trimethylamine–SO_3 complex. Ten percent Hya150 solution in DMF was mixed with TMA–SO_3 complex (Aldrich Chemical) and stirred for 48 h at 60 °C. SHya was obtained after the reaction by the method equal to the above-mentioned DMF–SO_3 complex method.

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.

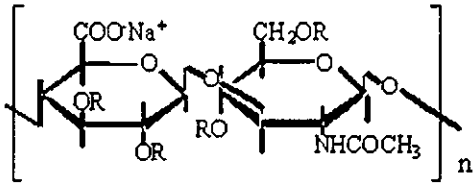
Cell culture. The rOB cells were isolated and cultured using the method described by Hamano et al [16]. rOB cells were cultured in a sterile tissue culture dish (NUNCLON) with the use of Dulbecco's modified Eagle's medium (DMEM, Nissui-seiyaku) supplemented with 10% fetal bovin serum (FBS, Gibco). Cultures 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

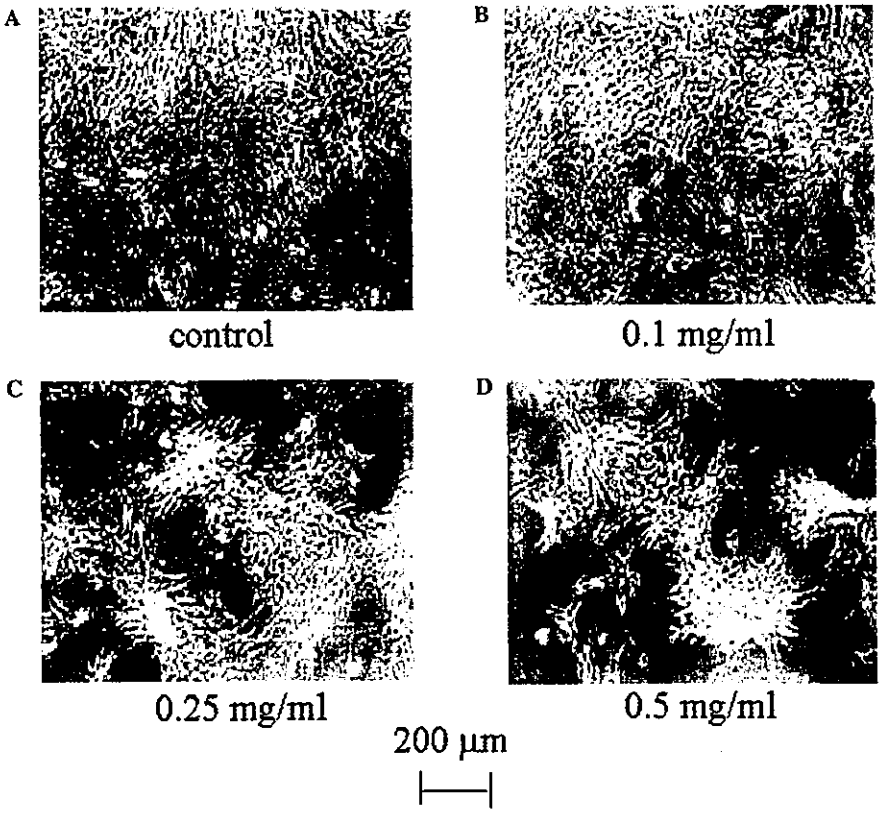


Fig. 2. Relationship between 2.1SHya concentration and rOB cell adhesion after 24 h. rOB cells were treated with various concentrations of 2.1SHya. (A) Control. (B) 0.1 mg/ml of 2.1SHya. (C) 0.25 mg/ml of 2.1SHya. (D) 0.5 mg/ml of 2.1SHya. Phase contrast micrographs. Scale bar 200 μm.

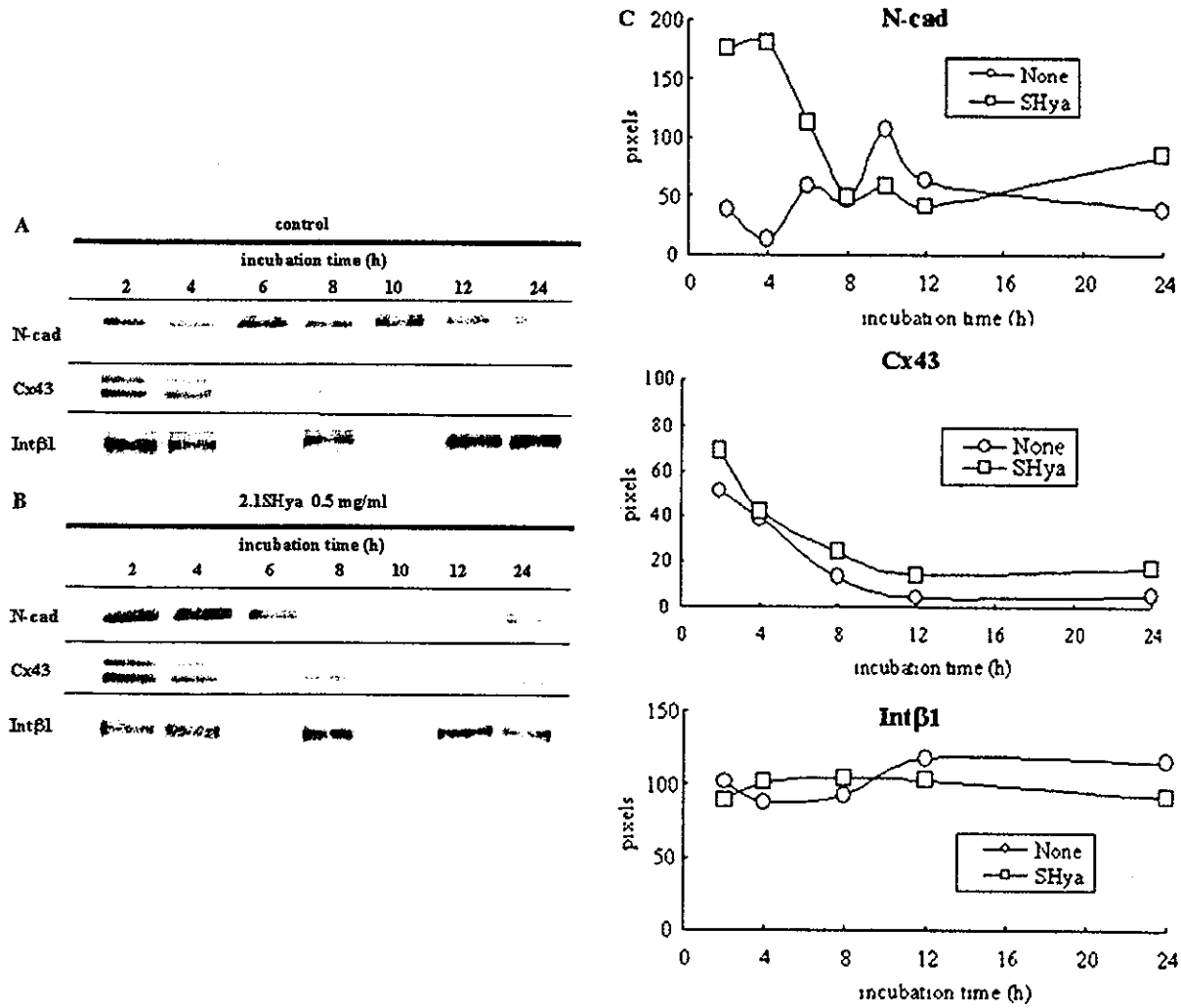


Fig. 3. Effect of SHya on adhesion protein expression in rOB cells. rOB cells were incubated with 2.1SHya for the times shown. Cells were lysed and proteins were separated by SDS–PAGE followed by Western blotting. (A) Without 2.1SHya (B) with 2.1SHya (C) quantification of band intensities was measured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

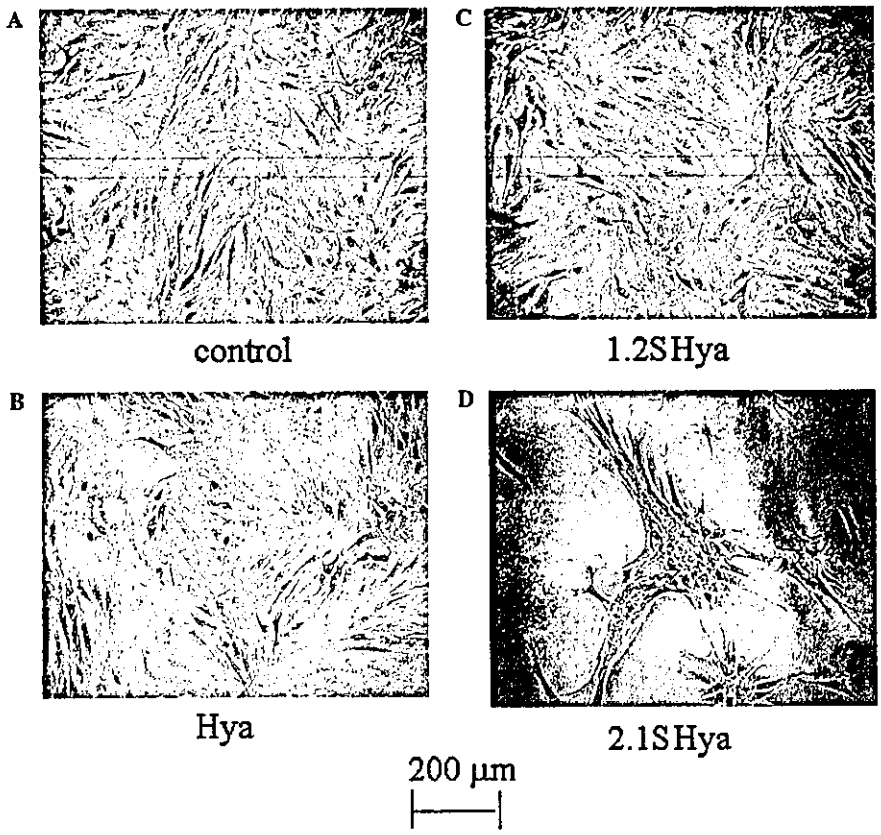


Fig. 4. Cell morphologies of rOB cells in the presence of 0.5 mg/ml Hya and SHya after 24 h. rOB cells were treated with Hya and varying DS of SHya. (A) Control, (B) Hya, (C) 1.2SHya, and (D) 3.4SHya.

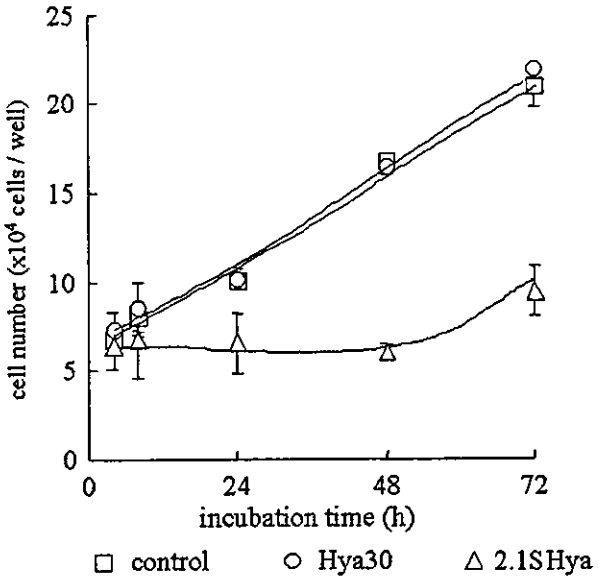


Fig. 5. Effect of 0.5 mg/ml Hya and SHya on the proliferation of rOB cells. rOB cells were treated with Hya and 2.1SHya for 72 h. The proliferation of rOB cells treated with Hya and 2.1SHya was determined. Values are means ± SD for four dishes.

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

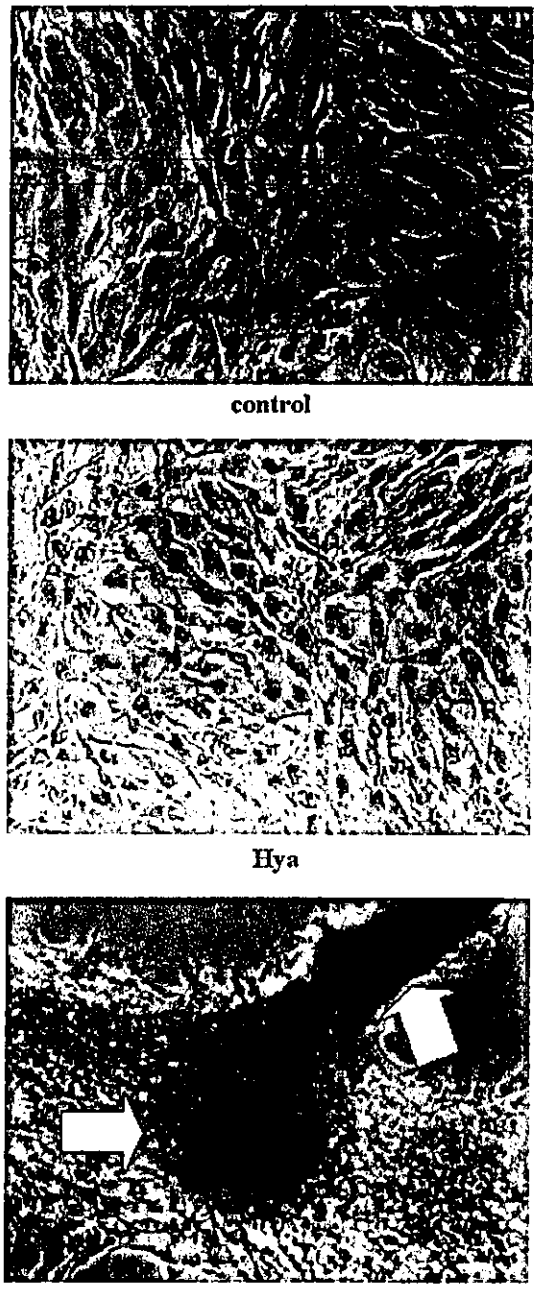


Fig. 6. Appearance of Azo-stained cultures of rOB cells in the presence of 0.5 mg/ml Hya and SHya after 24 h. rOB cells were treated with Hya and 2.1SHya for 24 h. rOB cells were stained by the Azo stain method.

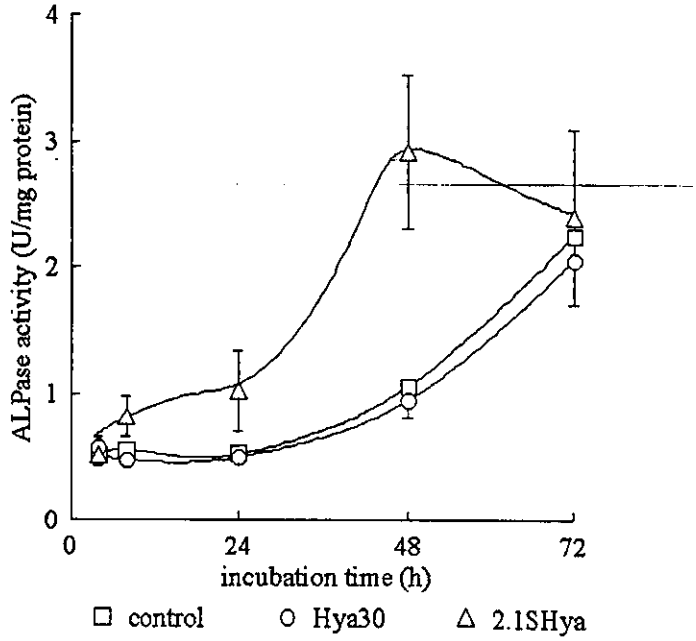


Fig. 7. Effect of 0.5 mg/ml Hya and SHya on the ALPase activity of rOB cells. rOB cells were treated with Hya and 2.1SHya for 72 h. The ALPase activity of rOB cells treated with Hya and 2.1SHya was determined. Values are means ± SD for four dishes.

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

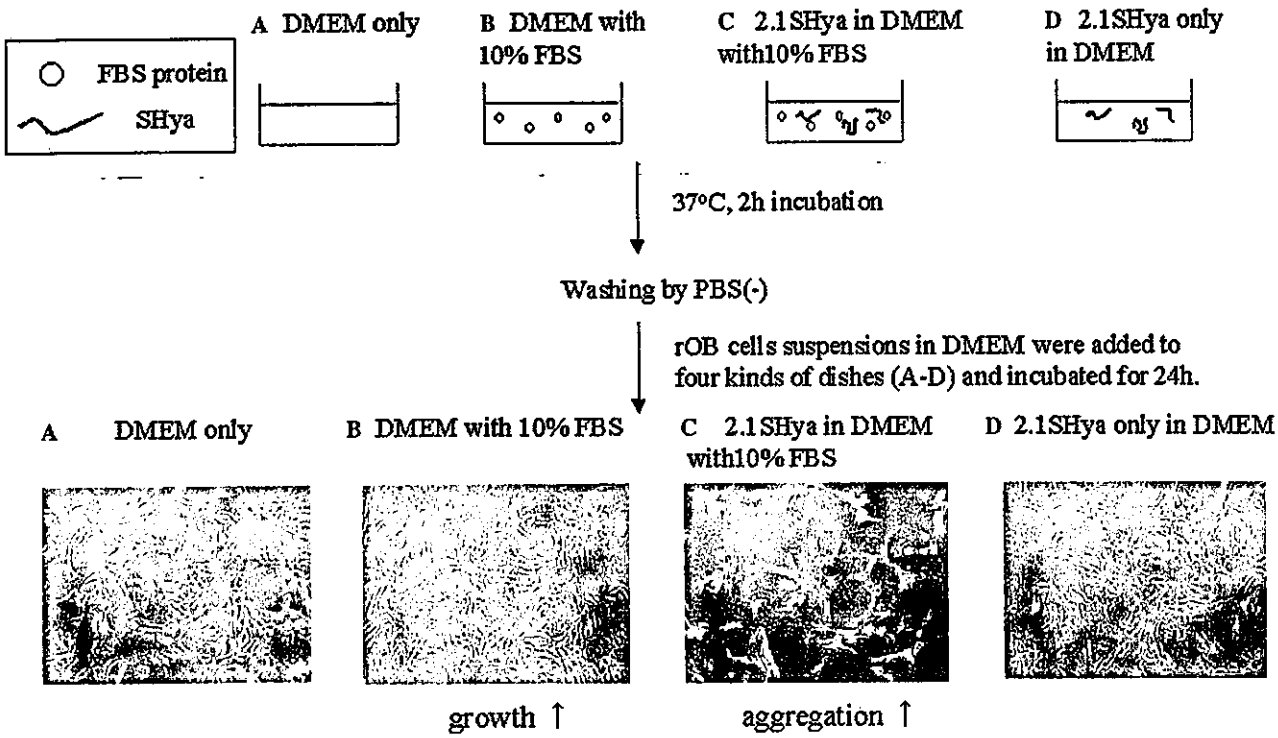


Fig. 8. Effect of FBS and 2.1SHya on cellular adhesion after 24 h. Four kinds of dishes were prepared as follows: (A) DMEM only, (B) DMEM with 10% FBS, (C) 2.1SHya in DMEM with 10% FBS, and (D) 2.1SHya only in DMEM into 35-mm tissue culture dish (NUNCLON) were incubated at 37 °C for 2 h under the 5%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

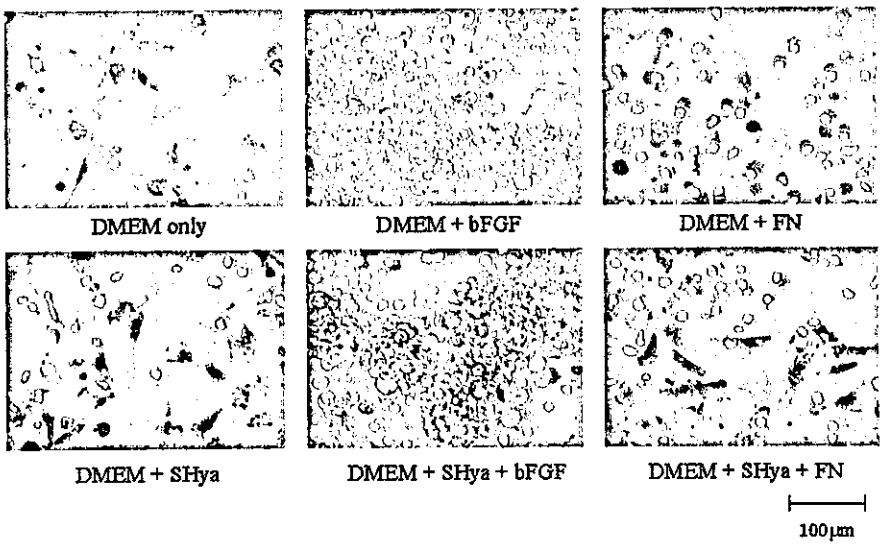


Fig. 9. Effect of serum component and 2.1SHya on cellular adhesion after 24 h. The cells were plated in serum free DMEM supplemented with FN, bFGF, and SHya, and incubated for 24 h at 37 °C with 5% CO_2.

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

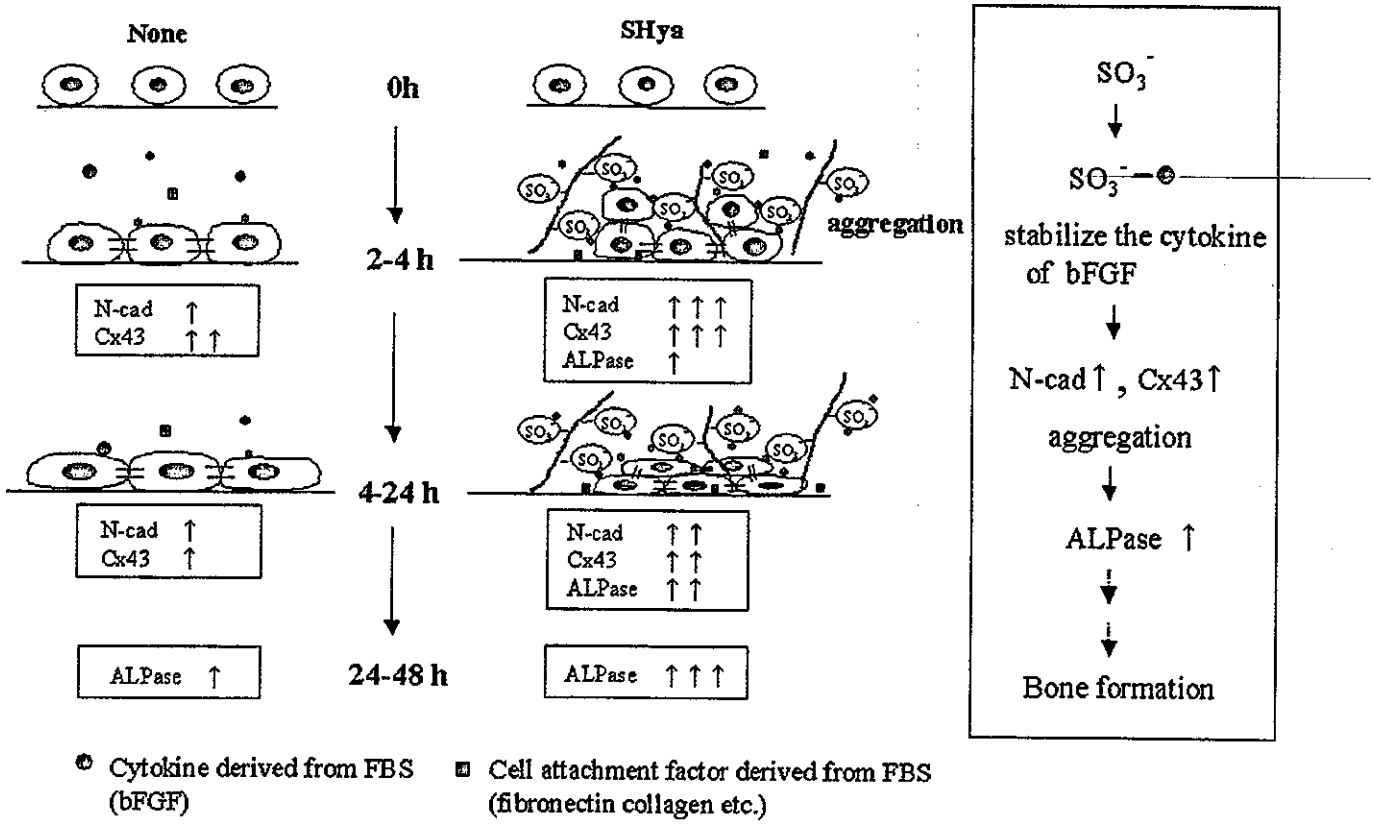


Fig. 10. SHya regulation of rat osteoblast cell differentiation.

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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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NHOst). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NHOst on it although the NHOst on it showed an enhancement in their differentiation level. On the other hand, NHOst on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NHOst was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NHOst on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NHOst to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NHOst on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using in vitro techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

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2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 50 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 8 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	$\frac{\text{ALP activity}}{\text{The cell number}}$ (ratio)	$\frac{\text{Ca amount}}{\text{The cell number}}$ (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in SBF. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC

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*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in SBF. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC

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Novel mechanism of tumorigenesis: Increased transforming growth factor-β1 suppresses the expression of connexin 43 in BALB/cJ mice after implantation of poly-L-lactic acid

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Abstract: Poly-L-lactic acid (PLLA) is a widely used promising material for surgical implants such as tissue-engineered scaffolds. In this study, we aimed to determine the *in vivo* effect of PLLA plates on the cellular function of subcutaneous tissue in the two mouse strains, BALB/cJ and SJL/J, higher and lower tumorigenic strains, respectively. Gap-junctional intercellular communication (GJIC) and the expression of connexin 43 (Cx43) protein were significantly suppressed, whereas the secretion of transforming growth factor-β1 (TGF-β1) level was significantly increased in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls. However, no significant difference in TGF-β1 secretion was observed between the SJL/J-implanted and SJL/J control mice. We found for the first time that a significant difference was observed between the two strains; thus, the PLLA increased the secretion of TGF-β1 and suppressed the mRNA expression of Cx43 at the earlier stage after implantation into the higher-tumorigenic strain, BALB/cJ mice. This novel mechanism might have a vital role in the inhibition of GJIC and promote the tumorigenesis in BALB/cJ mice. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 335–340, 2004

Key words: poly-L-lactic acid; gap-junctional intercellular communication (GJIC); connexin 43; transforming growth factor (TGF)-β; tumorigenesis

INTRODUCTION

The implantation of a biomaterial always induces a host inflammatory response. The extent and resolution of these responses have a vital role in determining the long-term success of implanted medical devices.[1–3] Poly-L-lactic acid (PLLA) is a widely used material for surgical implants and clinically as a bioabsorbable suture material.[4,5] Polyurethanes (PUs) have also been used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, and good abrasion resistance. Some adverse effects of the biomaterials, such as PLLA and PUs, have been reported in animal experiments. Long-term implants of PLLA produced tumorigenicity in rats.[6] Different kinds of PUs induced various tumor incidences in rats.[7] All tumors have been generally viewed as the outcome of disruption of the homeostatic regulation of the cellular ability to respond to extracellular signals, which trigger intracellular signal transduction abnormalities.[8] During the evolutionary transition from the single-cell organism to the multicellular organism, many genes appeared to accompany these cellular functions. One of these genes was the gene coding for a membrane-associated protein channel (the gap junction).[9] Gap-junctional intercellular communications (GJIC) 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 a cell- and development-specific manner.[10,11] GJIC also has an important role in the maintenance of cell homeostasis and in the control of cell growth.[12] So, the loss of GJIC has been considered to cause abnormal development and tumor formation.[13–15] Several tumor promoters have been shown to restrict GJIC by phosphorylation of connexin proteins, such as connexin 43 (Cx43), which is an essential

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protein to form the gap-junction channel.[16,17] We have hypothesized that the different tumorigenic potentials of PLLA and PUs are caused mainly by the different tumor-promoting activities of these biomaterials. Therefore, we investigated the effects of PLLA on the subcutaneous tissue between the two strains of female mice, BALB/cJ and SJL/J.

MATERIALS AND METHODS

Animals

Five-week-old female BALB/cJ and SJL/J mice were purchased from Charles River (Japan) and maintained in the animal center according to the animal welfare National Institute of Health Sciences guidance. All mice were fed with standard pellet diets and water *ad libitum*, before and after the implantation.

Implantation of PLLA

PLLA was obtained from Shimadzu Co. Ltd. as uniform plates. Implants (size: 20 × 10 × 1 mm, weight-average molecular weight 200,000) were sterilized using ethylene oxide gas before use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. The dorsal skin was shaved and scrubbed with 70% alcohol. Using an aseptic technique, an incision of approximately 2 cm was made; away from the incision, a subcutaneous pocket was formed by blunt dissection, and one piece of PLLA was placed in the pocket. The incision was closed with silk threads. In both strains, controls were obtained by sham operation and subsequent subcutaneous pocket formation. After surgery, the mice were housed in individual cages. After 30 days, mice from the implanted group were sacrificed, implanted materials were excised out, and subcutaneous tissues from the adjacent sites were collected for culture. At the same time, subcutaneous tissues were removed from the sites in the sham-operated controls that correlated with the implant sites.

Cell culture of subcutaneous tissues

The subcutaneous tissues were maintained in minimum essential medium supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C.

Scrape-loading and dye transfer (SLDT) assay

SLDT technique was performed by the method of El-Fouly et al.[18] Confluent monolayer cells in 35-mm culture dishes were used. After rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)], cell dishes were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR) in PBS (+) solution and were 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 transfer was monitored using a fluorescence microscope, equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan).

Western blot analysis

When cells grew confluently in 60-mm tissue culture dishes, all cells were lysed directly in 100 μL of 2% sodium dodecyl sulfate (SDS) gel loading buffer (50 m*M* Tris-HCl, pH 6.8, 100 m*M* 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The protein concentration of the cleared lysate was measured using the microplate BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). Equivalent protein samples were analyzed by 7.5% SDS–polyacrylamide gel electrophoresis. The proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). Cx43 protein was detected by anti-Cx43 polyclonal antibodies (ZYMED Laboratories, Inc., San Francisco, CA). The membrane was soaked with Block Ace (Yukijirusi Nyugyo, Sappro, Japan), reacted with the anti-Cx43 polyclonal antibodies for 1 h, and after washes with PBS containing 0.1% Tween20, reacted with the secondary anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase for 1 h. After several washes with PBS–Tween20, the membrane was detected with the ECL detection system (Amersham Pharmacia Biotech UK Ltd.).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Cx43 mRNA expression was verified by RT-PCR. Total cellular RNA was isolated from cultured cells in Trizol reagent (Life Technologies, Inc., Frederick, MD) following the manufacturer's instructions. The concentration of total RNA was determined using a UV spectrophotometer (Gene Quant; Pharmacia Biotech, Piscataway, NJ). cDNA was synthesized from 1 μg of total RNA by RT using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). 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) and 0.2 m*M* of each deoxynucleotide triphosphate. The sequence of the primer pairs were as follows: forward 5′-ACAGTCTGCCTTTCGCTGTAAC-3′ and reverse 5′-GTAAGGATCGCTTCTTCCCTTC-3′. The PCR cycle was as follows: initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 7 min. The amplified product was separated on 1.5% agarose gel and visualized with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME). For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene,

GAPDH. To amplify this gene, the following primer pairs were used: forward 5'-CCCATCACCATCTTCCAGGAGC-GAGA-3' and reverse 5'-TGGCCAAGGTCATCCATGA-CAACTTTGG-3'.

Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded onto 60-mm dishes. The conditioned medium was collected and obtained after the centrifugation at 1000 rpm for 2 min. The transforming growth factor (TGF)-β levels of the media were measured with commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN).

Cytokine treatment

Here, we used sham-operated BALB/cJ mice cells as a control. One hundred thousand cells were seeded onto 35-mm tissue culture dishes and cultured. After 4 h seeding in a 5% CO_2 atmosphere at 37°C, cells were treated with TGF-β1 (0, 2, and 10 ng/mL). Thereafter, SLDT and RT-PCR were performed. Purified human TGF-β1 was purchased from R&D Systems.

Statistical analysis

Student *t* test was used to compare the implanted samples with the controls. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

There are many known tumorigenesis-inducing factors. It was reported that many plastics induce malignant tumors when implanted subcutaneously into rats and mice.[19–22] PLLA shows slow degradation, and therefore has been applied as a biomaterial for surgical devices such as bone plates, pins, and screws. It was reported in different studies that polyetherurethane, polyethylene, and PLLA produced tumors in rats.[6,7,23–25] In our study, tumors were induced by PLLA plates in BALB/cJ mice at 100% incidence but not in SJL/J mice at the surrounding tissues of PLLA plates during a 10-month *in vivo* study. To understand the mechanisms of tumorigenesis induced by PLLA, we focused on the inhibitory effects on GJIC at the early stage of tumorigenesis. To assess functional GJIC, the SLDT assay was performed. Brand et al.[26] reported that BALB/cJ mice are a higher and SJL/J mice are a lower tumorigenic strain. Our present results showed that the GJIC was significantly inhibited in 1-month PLLA-implanted BALB/cJ mice cells compared with BALB/cJ controls (Fig. 1). In contrast, no significant difference was observed between the 1-month PLLA-implanted SJL/J mice and SJL/J controls (Fig. 1). The data also revealed that the dye migration was higher in control BALB/cJ mice than control SJL/J mice (Fig. 1). High responder to the tumorigenicity may be classified as animals that are easily suppressed in both GJIC function and the connexins expression. This perturbed gap junction is likely to have a major role in the PLLA-induced tumorigenesis. Gap junctions are also regulated by the posttranslational phosphorylation of the carboxy-terminal tail region on the connexin molecule. Phosphorylation of connexin molecules is closely related with the inhibition of GJIC.[27,28] Phosphorylation has been involved in controlling a broad variety of connexin processes that include trafficking, gathering/nongathering, degradation, and also the gating of gap channels. It was also reported that communication-deficient cells did not express the Cx43–biphosphorylated (P_2) isoform but cells with low gap-junction permeability showed detectable amounts of the Cx43–monophosphorylated (P_1) isoform.[16] To survey the cause, we examined the mRNA and protein expression of the Cx43 gene. Here, mRNA expression was suppressed in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls [Fig. 2(A)]. No significant difference was observed between the PLLA-implanted SJL/J mice and SJL/J controls [Fig. 2(B)]. We also found that the total level of protein expression such as unphos-

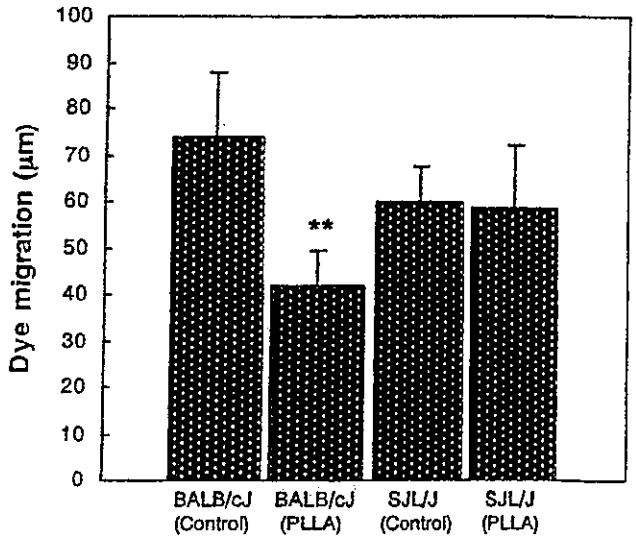

Figure 1. Statistical analysis of the SLDT assay. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice cells compared with BALB/cJ controls. $^{**}p < 0.01$.

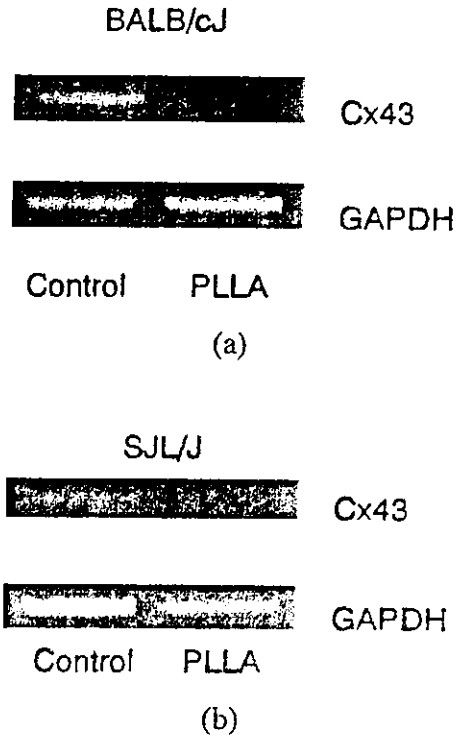

Figure 2. mRNA expression of Cx43 by RT-PCR analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. SYBR Green I stained PCR products after agarose gel electrophoresis showed that (A) mRNA expression was suppressed in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls, and (B) no significant difference was observed between the PLLA-implanted SJL/J mice and SJL/J controls.

phorylated (P_0), P_1, and P_2 levels were significantly decreased in PLLA-implanted BALB/cJ mice compared with the control (Fig. 3). Asamoto et al.[29] reported that tumorigenicity was enhanced when the expression of Cx43 protein was suppressed by the anti-sense RNA of Cx43. A similar tendency was also observed in our study where the protein expression might be inhibited via down-regulation of the mRNA level. The genetic alteration and posttranslational

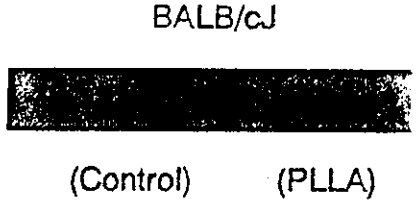

Figure 3. Protein expression of Cx43 by Western blot analysis. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Total level of protein expression such P_0, P_1, and P_2 levels were significantly decreased in PLLA-implanted BALB/cJ mice compared with the controls.

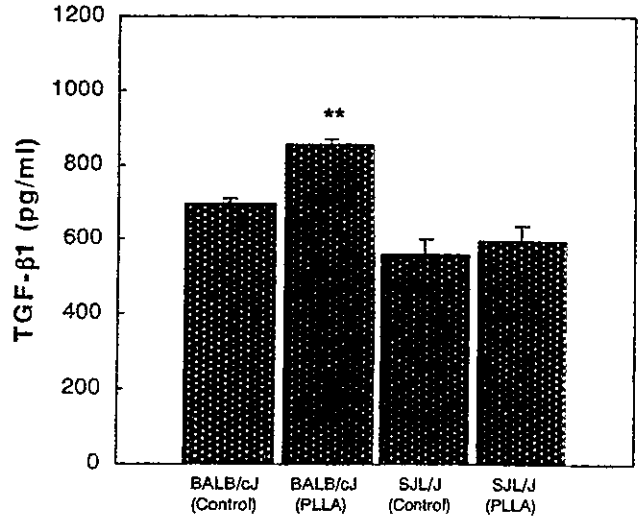

Figure 4. Statistical analysis of TGF-β1 cytokine assay by ELISA. In both the implanted and sham-operated controls, three mice of each strain were sacrificed after 30 days. Results shown are representative of two independent experiments. Secretion of the TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ mice compared with BALB/cJ controls. $^{**}p < 0.01$.

modification in the Cx43 protein was shown to be involved in impaired GJIC and could be associated with tumorigenesis. Therefore, it is suggested that the inhibitory effect of PLLA on GJIC might be caused by the alteration in the Cx43 protein, causing enhancement of tumorigenesis. Moreover, Moorby and Patel[30] reported a direct action of the Cx43 protein on cell growth that was mediated via the cytoplasmic carboxyl domain.

Because TGF-β1 inhibits GJIC by decreasing the phosphorylated form of Cx43[31] and the phosphorylation of Cx43 has been implicated in gap-junction assembly and gating events,[16,27,32] we hypothesized that TGF-β1 might have an important role on PLLA-implanted BALB/cJ mice. Figure 4 clearly demonstrates that the secretion of the TGF-β1 level was significantly increased in PLLA-implanted BALB/cJ subcutaneous tissue in comparison with those from BALB/cJ control mice. No significant difference was found in the secretion of TGF-β1 between the SJL/J implanted and SJL/J control mice. TGF-β2 and TGF-β3 cytokine assay revealed no significant difference in TGF-β2 secretion and TGF-β3 was below the detection level (data not shown). So we performed an *in vitro* study, which showed that the intercellular communication and the mRNA expression of Cx43 were significantly suppressed in BALB/cJ control cells when treated with TGF-β1 [Fig. 5(A,B)].

In conclusion, we suggest that increased secretion of TGF-β1 (Fig. 4) suppressed expression of the gap-junctional protein Cx43 (Fig. 3) at the earlier stage after implantation of PLLA in BALB/cJ mice, resulting in